EPIDEMIOLOGY, DETECTION, MOLECULAR TYPING AND CONTROL OF PORCINE COLONIC SPIROCHAETOSIS (*Brachyspira pilosicoli*)

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DECLARATION

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Abstract

Brachyspira pilosicoli is a Gram negative anaerobic intestinal spirochaete which is the aetiological agent of porcine colonic spirochaetosis (PCS). PCS is prevalent in the United Kingdom and many other countries. This study aimed to determine risk factors associated with PCS, assess detection procedures, investigate the genetic variation among isolates from porcine colitis outbreaks and evaluate the efficacy of disinfectant-sanitisers against *B. pilosicoli in vitro*. An epidemiological study was carried out using data from pig farms with and without infectious colitis to determine possible risk factors. Categorical variables including a wide range of housing and management factors were analysed. The major factors associated with infectious colitis involving *B. pilosicoli* were: source of breeding stock replacements ($p<0.045$), presence of concurrent infection with *Streptococcus suis* on the farm ($p<0.038$), and bedded floors ($p<0.007$). Fully slatted floors were identified as a protective factor ($p<0.007$).

The diagnostic potential of immunomagnetic separation (IMS) for detection of *B. pilosicoli* and *B. hyodysenteriae* from faecal samples was investigated. The sensitivities of the IMS (direct and indirect methods) using polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) were evaluated and compared with the standard diagnostic method namely direct culture, and polymerase chain reaction (PCR). IMS using PAbs did not improve the diagnostic sensitivity. IMS using MAbs did not increase diagnostic sensitivity when performed with the recommended washing steps. However, IMS performed without the washing steps improved the sensitivity for detection of *B. pilosicoli* and had higher sensitivity than direct culture. PCR was more sensitive than the IMS using MAbs (performed with the recommended washing steps). IMS using MAbs shows potential to improve detection of *Brachyspira* spp. but further work on a method that avoids significant loss of target cells during the washing steps is required.

The genetic diversity of single and multiple isolates from pig farms, two dog isolates and one human isolate of *B. pilosicoli* was investigated by arbitrarily primed PCR (AP-PCR). One isolate of the different species *B. hyodysenteriae* was included as an outgroup. AP-PCR was optimised using four primers individually. The generated data was subjected to analysis by distance method and parsimony method. The DNA banding patterns of *B. hyodysenteriae* and *B. pilosicoli* were clearly differentiated by each primer. Phylograms were generated from DNA fragment data for each primer and for pooled data (fragment data of the four primers) to give more robustness to the analysis. The high genetic variation shown by some of the multiple isolates from the same farm suggested that infections by *B. pilosicoli* in UK farms might not be caused by a single (clonal) genotype.

Seven disinfectant-sanitisers were tested for their efficacy against six isolates of *B. pilosicoli*. The products were commercially available and included four different chemical groups: quaternary ammonium group, tar-organic acid group, caustic soda group and peroxide group. Serial 10-fold dilutions of the products were prepared and challenged with an inoculum of 10^5 bacterial cells of *B. pilosicoli*. To determine the importance of thorough cleaning of farm buildings in relation to the potential of efficacy of disinfectant agents, these products were tested with and without presence of organic matter (as sterile pig faeces). Contact times of 15, 30, 45 and 60 minutes between the disinfectant agents and *B. pilosicoli* were evaluated. No differences were

observed between contact times or between isolates. In the absence of organic matter, the highest efficacies were obtained by DSC-1000 and Long Life with 64 and 50.0 % efficacy at 1:10000 dilution, followed by Ambicide with 43.0 % efficacy at the same dilution (1:10000). In the presence of organic matter the best results were achieved by DSC-1000 and Long Life both with 14.0 % efficacy at dilution 1:1000. The presence of organic matter had a negative effect on the efficacy of the products evaluated against *B. pilosicoli* ($P<0.006$), confirming the importance of thorough cleaning prior to disinfection. Heavy Duty and Virkon S were the most affected by the presence of organic matter with efficacies at the level of 7.0 and 0.0 %, respectively, at the 1:100 dilution. The chemical groups that showed the best performance in the presence of organic matter were the quaternary ammonium and tar-organic acids groups in particular the products DSC-100, Long Life, Ambicide and Farm Fluid.

Chapter one

LITERATURE REVIEW

1. Introduction

1.1. Spirochaetes

The two families *Spirochaetaceae* and *Leptospiraceae* form the order *Spirochaetales*. The family *Spirochaetacea* includes the species of the genera *Treponema, Spirochaetae, Borrelia, Brachyspira, Brevinema* and *Cristispira*, and the family *Leptospiraceae* includes the genera *Leptospira* and *Leptonema* (Fig. 1.1). Although, spirochaetes share many characteristics, they represent a diverse group of pathogenic and non-pathogenic organisms.

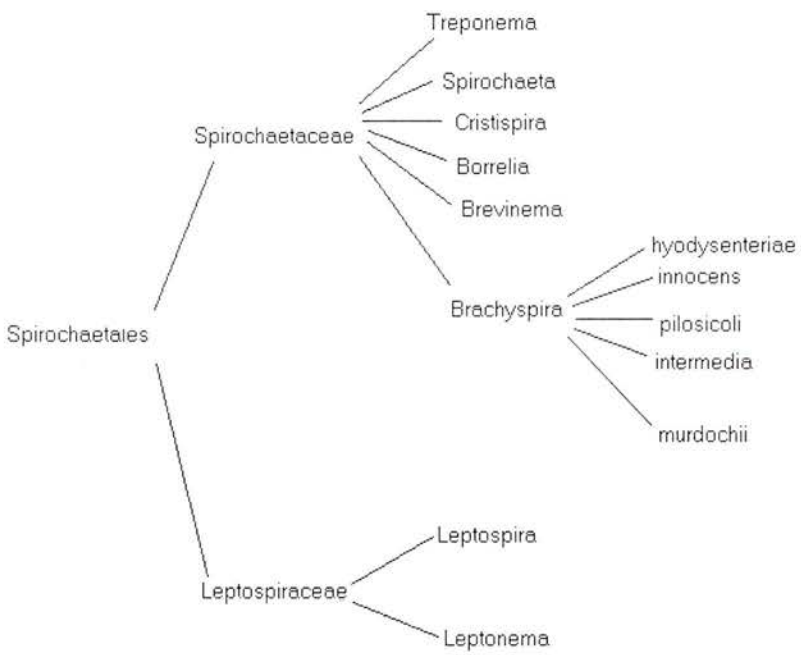

Fig. 1. Phylogenetic classification of the order Spirochaetales

There have been many advances in the field of spirochaetes, particularly in intestinal spirochaetes in recent years as a result of advances in molecular biology and phylogenetic studies. For instance, the genetic analysis of intestinal spirochaetes by

DNA-DNA reassociation, polyacrylamide gel electrophoresis (PAGE) of proteins, restriction endonuclease analysis of DNA, and 16S rRNA sequence analysis determined that *Treponema hyodysenteriae* and *Treponema innocens* were not actually members of the genus *Treponema*. These spirochaetes were initially renamed as *Serpula hyodysenteriae* and *Serpula innocens* (Stanton *et al*, 1991), however since *Serpula* was the name of a fungal genus, the genus was renamed *Serpulina*, and the species *Serpulina hyodysenteriae* and *Serpulina innocens*, respectively (Stanton *et al,* 1991; Stanton, 1992).

In 1996, *Serpulina pilosicoli* (formerly *Anguillina coli*) was recognised as a new species after DNA-DNA comparisons with the species *S. hyodysenteriae* and *S. innocens* (Trott *et al*, 1996c). Further members of this genus were identified as *Serpulina intermedia, Serpulina murdochii, Serpulina alvinipulli* and *Serpulina 'canis'*. Late in the 1990s, the genus *Serpulina* was included in the genus *Brachyspira* as result of phylogenetic studies based on complete 16S ribosomal DNA sequences (Ochai *et al*, 1997). *Brachyspira* is a recently defined genus including commensal and pathogenic species, which infect a wide range of hosts.

1.2. Characteristics of spirochaetes

The morphological characteristics of spirochaetes make them unique; they possess a spiral (serpentine) shaped body resembling little snakes that are surrounded by periplasmic flagella, which provide motility (Canale-Parola, 1978). These characteristics in particular distinguish spirochaetes from other bacteria. The size of the spirochaetal body and the number of periplasmic flagella vary between genera and also between species. These morphological characteristics along with other features are used to differentiate the genera and species of spirochaetes (Fig. 1.2).

Fig. 1.2 . Characteristic morphology of intestinal spirochaetes

Intestinal spirochaetes are classified as Gram negative anaerobic but oxygen tolerant unicellular bacteria that are found either colonising the digestive tracts (from the mouth to the rectum) of various animals and humans, or as free-living organisms. For instance, the species *Treponema denticola* and *Treponema pectinovorum* are found in the oral cavity of humans. Other spirochaetes like *T. succinifaciens* inhabit the colon of pigs.

Some spirochaetes also are part of complex epidemiology, i.e., species of the genus *Borrelia* are transmitted into humans by a tick vector to cause the diseases known as Lyme disease and relapsing fever (Barbour and Hayes, 1986). Moreover, there are some spirochaetes that have been identified but it has not been possible to grow them *in vitro*

e.g. the genus *Cristispira* (Paster *et al,* 1996). As progress in research on the field of spirochaetes has been achieved, the probability of discovering species affecting unusual hosts seems to be high.

Depending on their physiological characteristics, spirochaetes are classified as strictly obligate anaerobes (e.g. *Treponema* species), oxygen tolerant anaerobes (e.g. *Brachyspira* species), free-living organisms (e.g. *Spirochaeta aurantia, Spirochaetae zuelzerae* or *Spirochaetae stenostrepta*), or affecting multiple host species (e.g. *B. pilosicoli*). Other spirochaetes such as the genera *Leptospira* and *Borrelia* are aerobic and microaerophilic, respectively (Baseman, 1990). Further variation is seen in their habitat (intestinal tract) and pathogenicity.

1.3. Intestinal spirochaetes as commensals or pathogens.

Intestinal spirochaetes are species identified within the genus *Treponema* and the genus *Brachyspira* (formerly *Serpulina*) (Ochai, 1997). The microhabitat and adaptability of intestinal spirochaetes correlates with their characteristics of growth and pathogenicity, for instance, the *Brachyspira* species are found in close contact with the intestinal mucosa, which may explain their oxygen tolerance. In contrast, the *Treponema* species that inhabit the intestine grow in an environment away from the intestinal or rumen mucosa where little oxygen is present which may explain their strict anaerobiosis (Stanton, 1997).

Commensal intestinal spirochaetes may perform important functions. For instance, the rumen of the bovine is colonised by two non-pathogenic *Treponema* species that participate in the degradation of plant polysaccharides such as pectin, xylan, arabinogalactan and starch, these species are identified as *Treponema bryantii*

(Stanton and Canale-Parola, 1980) and *Treponema saccharophilum* (Paster and Canale-Parola, 1985). In the pig, the non-pathogenic species *Treponema succinifaciens* (Harris and Kinyon, 1974) is found colonising the large intestine, mainly the colon.
Intestinal spirochaetes are most closely associated with pigs, and are mainly members of the genus *Brachyspira* that includes commensal and pathogenic species (Table 1.1). The recognised pathogenic species *Brachyspira hyodysenteriae*, the aetiological agent of swine dysentery (SD) (Taylor and Alexander, 1971), and *Brachyspira pilosicoli,* the aetiological agent of a pathological condition of the colon of pigs known as intestinal spirochaetosis (IS) (Taylor, 1980), or porcine colonic spirochaetosis (PCS) (Duhamel *et al,* 1995a). PCS is now the term internationally recognised to refer to this disease, and will be used for consistency throughout. *B. pilosicoli* infection has been reported in a wide range of hosts such as dogs (Lee and Hampson, 1994; Duhamel *et al* 1995a), chickens and other avians (Dwars *et al,* 1992; Trott, *et al,* 1995; McLaren *et al,* 1997; Webb *et al,* 1997), humans (Houvin-Hougen *et al,* 1982; Cooper *et al,* 1986; Jones *et al* 1986; Surawicz *et al,* 1987; Gebbers *et al* 1987; Lee and Hampson, 1994), non-human primates (Takeuchi *et al* 1974), guinea pigs (Vanrobaeys *et al*, 1998).

Brachyspira innocens (Stanton, 1992) is found in the colon of pigs and is regarded as non-pathogenic. There are two other species of intestinal spirochaetes for which pathogenicity in pigs is not clear yet. These species have been designated as *'Brachyspira-Serpulina' intermedia* (Stanton *et al,* 1997), which has been suggested to be involved in a mild colitis in pigs (Fellström and Gunnarsson, 1995), and also shown to be pathogenic for chickens (McLaren *et al,* 1997). While *'Brachyspira-Serpulina' murdochii* (Stanton *et al,* 1997) that has been found colonising the intestines of swine and rats in the absence of disease. *'Brachyspira-Serpulina' intermedia* and *'Brachyspira-Serpulina' murdochii* have not been formally homologated into the genus *Brachyspira.*

As interest in the field of intestinal spirochaetes has grown, further species have been identified recently as pathogens in animal species other than pigs. These new species

have been named *Serpulina alvinipulli* (Stanton *et al,* 1998), which was shown to be pathogenic for chickens, and the provisionally designated *Serpulina canis* (Duhamel *et al,* 1998). A role for the latter as a causative agent of disease remains unclear since most of the specimens were isolated from healthy dogs (Duhamel *et al*, 1998).

In humans the intestinal spirochaete *Brachyspira aalborgi* was originally reported as non-pathogenic (Hovind-Hougen *et al*, 1982). However, recent reports have demonstrated that this species is associated with intestinal pathology and can be considered as cause of human intestinal spirochaetosis (Mikosza *et al,* 1999). As interest continues, there is no doubt that other spirochaete species have not been identified as yet due to the fact that they have not been cultivated (Stanton *et al,* 1997).

Table 1.1. Recognised species of intestinal spirochaetes

Genus and species	Host	Location	Pathogenicity
Brachyspira hyodysenteriae	pigs	large intestine	Pathogenic
B. innocens	pigs	large intestine	non-pathogenic
'Brachyspira-Serpulina' intermedia	pigs	large intestine	pathogenic
'Brachyspira-Serpulina' murdochii	pigs, rats	large intestine	non-pathogenic
B. pilosicoli	pigs, dogs, chickens and other avians, guinea pigs, non-humans primates, humans	large intestine	pathogenic
Serpulina alvinipulli	chickens	caecum	pathogenic
Serpulina canis	dogs	large intestine	non-pathogenic?
B. aalborgi	humans	large intestine	non-pathogenic?
Treponema bryantii	bovine	rumen	non-pathogenic
T. saccharophilum	bovine	rumen	non-pathogenic
T. succinifaciens	pigs	large intestine	non-pathogenic

Modified from Stanton (1997)

2. Porcine intestinal spirochaetes

The first report of intestinal spirochaetes affecting pigs was in 1921 in the USA, and the pathological condition was named swine dysentery. After intensification of pig production in many countries SD became more frequent. In the early 1970s this intestinal spirochaete was isolated from faecal samples of affected pigs and grown *in vitro* (Taylor and Alexander, 1971), and soon after, the disease was reproduced according to the Koch's postulates. This microorganism was then named *Treponema hyodysentariae* by Harris *et al.* (1972), and it is the best characterised intestinal spirochaete.

Swine dysentery is a disease characterised by severe mucohaemorragic diarrhoea, inflammation, necrosis and excess of mucus production in the large intestine (Taylor and Alexander, 1971; Harris *et al*, 1972). This disease is one of the most important intestinal disorders in pigs with a detrimental economic effect on the pig industry in many countries. *B. hyodysenteriae* is the most infectious and the best understood disease within the intestinal spirochaetal infections in pigs. Lesions in the large intestine of pigs caused by *Brachyspira hyodysenteriae* are severe and characteristic (Harris and Lysons, 1992). PCS is less severe than SD, and it is characterised by a mild greyish diarrhoea (Taylor *et al*, 1980; Lemcke and Burrows, 1981) in pigs between 6 and 12 weeks of age (Duhamel, 1995a; Fellström and Gunnarsson, 1995). PCS is also world wide distributed and reports from various countries have been published, including Australia (Hampson, 1991), Canada (Girard *et al,* 1995), Denmark (Jensen, 1995), the UK (Taylor, 1984; Thomson *et al,* 1998), USA (Egan *et al.,* 1982; Andrews and Hoffman, 1983), Poland (Binek and Szynkiewickz, 1984), Sweden (Fellström *et al,* 1996), Finland (Heinomen, 2000), Brazil (Barcellos, 2000). The economic importance of PCS is the time delayed to market as a consequence of poor performance of affected pigs (Duhamel *et al,* 1995a).

B. pilosicoli infection is also reported to occur in humans (as mentioned above), mainly in defined populations such as individuals who are immunocompromised by HIV and homosexual males in Western communities (Cooper *et al,* 1986; Surawicz *et al,* 1987), certain ethnic groups such as Australian aborigines (Lee and Hampson, 1992), and in developing communities (Lee and Hampson 1994). Further *B. pilosicoli* has been recorded as a cause of disease in dogs (Duhamel *et al,* 1998). Although, *B. pilosicoli* affects a wide range of hosts, cross-species transmission has yet to be fully evaluated.

2.1. Differentiation of porcine intestinal spirochaetes

It is evident that intestinal spirochaetes are a group of morphologically similar microorganisms that includes pathogenic and non-pathogenic species. Due to identification of new pathogenic species in recent years, methods of differentiation between and within species are therefore important for diagnosis, epidemiological purposes, and phylogenetic studies. This is becoming increasingly important since it has been reported that some species show intermediate biochemical reactions (Hommez *et al,* 1998; Thomson *et al,* 2001), thus, accurate identification is necessary to assign significance.

2.2. Morphological and phenotypic differentiation of intestinal spirochaetes

Morphological characteristics of intestinal spirochaetes have been used to distinguish the different species using electron micrographs to assess the ultrastucture of the bacterial cells. Differences have been observed in dimensions of the bacterial cells and the number of flagella. *B. hyodysenteriae* and *B. innocens* have between 7 and 14 periplasmic flagella at each end, '*Brachyspira-Serpulina' intermedia* and *Brachyspira-Serpulina' murdochii* have between 12-14 and 11-13 flagella at each end, respectively, and the shape of the end of the cell is blunt (Harris *et al,* 1972; Hovind-Hougen *et al,* 1990; Lee *et al,* 1993a; Stanton *et al,* 1997). The cells of *B. pilosicoli* have between 4

and 7 periplasmic flagella at each end and pointed ends (Dettori *et al,* 1987; Lee *et al,* 1993a; Trott *et al,* 1996b). A lattice structure on the end of cells of *B. pilosicoli* isolated from pigs, humans and dogs has been described (Turek and Meyer, 1977; Hovind-Hougen *et al,* 1990), this structure may play a role in the ability of *B. pilosicoli* to attach to the intestinal epithelial cells by one end.

Regarding the dimensions of porcine intestinal spirochaetes, there are slight differences between the species (Table 1.2).

Table 1.2. Morphological characteristics of intestinal spirochaetes

Genus and species	Length (μm)	Width (μm)	Number of flagella at each end	Reference
B. hyodysenteriae	5.9-12.9	0.29-0.40	7-14	Lee *et al* (1993a) Trott *et al* (1996c)
B. innocens	5.3-14.1	0.25-0.40	7-14	Lee *et al* (1993a) Trott *et al* (1996c)
B. pilosicoli	5.2-11	0.19-0.30	4-7	Lee *et al* (1993a) Trott *et al* (1996c)
'Brachyspira- Serpulina' intermedia	7.5-10	0.35-0.45	12-14	Stanton *et al* (1997)
'Brachyspira- Serpulina' murdochii	5-8	0.35-0.4	11-13	Stanton *et al* (1997)
B. aalborgi	2-6	0.2	4	Hovind-Hougen *et al* (1982)

B. pilosicoli is slightly shorter than *B. hyodysenteriae*, *B. innocens* and *'Serpulina-Brachyspira' intermedia*. While only *'Serpulina-Brachyspira' murdochii* is shorter and also slightly thinner. However, there are reports showing that intestinal spirochaetes isolated from chickens with diarrhoea were indistinguishable from *B. hyodysenteriae* and *B. innocens* in terms of ultrastructure (Swayne *et al,* 1995). Further, the morphology of intestinal spirochaetes may be affected by culture conditions since spherical bodies could be observed in unfavourable conditions (Swayne *et al,* 1995).

Intestinal spirochaetes are regarded as fastidious, difficult to isolate and slow growing (Duhamel and Jones, 1994). Despite this, culture demonstrates one of the most

important differences between the species of intestinal spirochaetes, which is the type of haemolysis produced on blood agar (BA). *B. hyodysenteriae* produces a strong or complete β-haemolysis (Binek and Szynkiewicz, 1984; Fellström and Gunnarsson, 1995) whereas other porcine intestinal spirochaetes including *B. innocens, B. pilosicoli,* '*Serpulina-Brachyspira' intermedia* and '*Serpulina-Brachyspira' murdochii* are regarded as weakly β-haemolytic intestinal spirochaetes (WBHIS). *B. pilosicoli* has been shown to grow faster in liquid medium with a doubling time of 1-2 hrs compared to other intestinal spirochaetes such as *B. hyodysenteriae* which has doubling time of 3-5 hrs (Trott *et al,* 1996c), suggesting other physiological differences.

Haemolysis together with biochemical reactions such as indole production, hippurate hydrolysis activity, α and β-glucosidase, and α-galactosidase activities form the basis for differentiation of intestinal spirochaetes. Based on those characteristics intestinal spirochaetes have been classified into four clearly defined groups I to IV. Of these, group III is sub-divided into three sub-groups IIIa, IIIb and IIIc (Fellström and Gunnarson, 1995). As new species of porcine intestinal spirochaetes, particularly *'Brachyspira-Serpulina' intermedia* and *'Brachyspira-Serpulina' murdochii* were identified more recently (Stanton *et al,* 1997), the classification of Fellström and Gunnarsson (1995) was amended. Biochemical variations among some of the groups have been recognised and newly named species were matched to the groups to which they belonged (Fellström *et al,* 1999). *B. hyodysenteriae* (Group I) is characterised by strong β-haemolysis, positive indole reaction, positive α-glucosidase activity, and lack of α-galactosidase activity; indole production was thought to be associated with pathogenicity but indole negative isolates have been found in Belgium (Hommez *et al,* 1998), Germany and Canada (Fellström *et al,* 1999), and are thought to be also pathogenic. *'Brachyspira-Serpulina' intermedia* (Group II) produces weak β-haemolysis, positive indole production and β-glucosidase, and negative hippurate hydrolysis and α-galactosidase. *'Brachyspira-Serpulina' murdochii* (Group IIIa) produces weak β-haemolysis, negative indole production, hippurate hydrolysis and

α-galactosidase but positive β-glusosidase. *B. innocens* (Group IIIb and IIIc) produces weak β-haemolysis, negative indole production and hippurate hydrolysis, and positive α-galactosidase and β-glucosidase. *B. pilosicoli* (Group IV) produces weak β-haemolysis, negative indole production and β-glucosidase but positive hippurate hydrolysis activity and α-galactosidase though some isolates lack of α-galactosidase (Fellström *et al,* 1999). From this classification a distinction between *B. pilosicoli* (group IV) and other intestinal spirochaetes was the absence of indole production, strong hippurate hydrolysis and the lack of β-glucosidase activity. The biochemical characteristics of *B. pilosicoli* may be useful in the identification of this particular spirochaete (Trott *et al,* 1996b), since it has been shown that more than one type of spirochaete could be found in the same herd (Fellström and Gunnarsson, 1995), atypical biochemical reactions can be observed in the laboratory from a single sample. Interpretation of biochemical results should therefore be taken carefully, and further investigations may be needed to corroborate the identity of the microorganisms (Hommez, *et al,* 1998).

Another tool for differentiation of porcine intestinal spirochaetes is serotyping which consists of typing lipopolysacharides (LPS). This was first applied in the late 1970s when four serotypes of *B. hyodysenteriae* were recognised by hyperimmune sera against the whole bacterial cells raised in rabbits (Baum and Jones, 1979). By 1984 another three serotypes were discovered (Lemcke and Bew, 1984). Soon after a further three serotypes were reported (Mapother and Jones, 1985). In the serotyping differentiation of porcine intestinal spirochaetes there was high cross-reactivity between the different serotypes. Thus, a similar method as for serotyping of *Leptospira interrogans* was adopted in which serogroups were created using the type strain for each serogroup, and isolates showing a positive reaction within that serogroup would form a serovar (Hampson *et al*, 1989). It was also realised that there was antibody cross-reactivity between *B. hyodysenteriae* and *B innocens* on serological tests such as indirect hemagglutination (IHA), passive haemolysis (PH), agglutination and microagglutination

test (MAT). Cross-reactivity was improved by boiling the antigen before use in MAT which clearly differentiated the serotypes of *B. hyodysenteriae*, and between isolates of *B. hyodysenteriae* and *B. innocens* (Diarra *et al,* 1995). By 1991, there were 9 serotypes of *B. hyodysenteriae* identified as A to I (Li *et al,* 1991; Hampson, 1990). Eight of the serotypes of *B. hyodysenteriae* have been identified in Australia (Combs *et al,* 1992), and the serotypes H and I were isolated from Canada and correspond to the type strains FM 88-90 and FMV 89-3323, respectively (Li *et al,* 1991). The list of serogroups has been increased by the identification of two more serogroups. The serogroups are represented as A strain B78; B strain WA1; C strain B169; D strain A1; E strain WA6; F strain VS1; G strain Q16; H strain Vic2; I strain NSW1; J strain FM88-90 and K strain FMV89-3323 (Hampson *et al,* 1997).

Due to high antibody cross-reactivity between *Brachyspira* spp., specific differentiation of intestinal spirochaetes by serotyping methods has been poor. Attempts to increase the specificity by adsorbing antisera with heterologous species of intestinal spirochaetes has been done but the antibody titre reduced significantly (Lysons and Lemcke, 1983).

In contrast to *B. hyodysenteriae,* serogroups of *B. pilosicoli* have not been defined due to the lack of full characterisation of the antigenic features of this particular species. However, by analysis of the outer envelope of 8 strains of *B. pilosicoli* it has been found that the lipo-oligosaccharides (chemical molecules composing the outer envelope structure) were antigenically distinct from each other, suggesting that this spirochaete may be antigenically diverse (Lee and Hampson, 1999).

Several antisera have been produced for the purpose of diagnosis of SD but cross-reaction has been observed between *B. hyodysenteriae* and *B. innocens* due to the sharing of periplasmic flagella antigens (Miller *et al,* 1988; Smith *et al,* 1990). In another study, four monoclonal antibodies (MAbs) recognised both *B. hyoysenteriae* and *B. innocens* in plate and rapid dot ELISA. Although, no cross-reactivity was observed with any other Gram negative bacteria the characterisation of four polypeptide antigens

of molecular weight 26 to 45 kDa common to *B. hyodysenteriae* and *B innocens* support previous reports of similarity of structural epitopes between *Brachyspira* spp. (Achacha and Mittal 1996).

A monoclonal antibody specific for *B pilosicoli* has been produced in mice using cell envelope protein preparations (Lee and Hampson 1995). This MAb of IgM subclass showed specificity in Western blots to a 29 kDa cell envelope protein characteristic of *B. pilosicoli* and '*Serpulina-Brachyspira' jonesii* but no reaction was observed to cell envelope proteins of any other of the spirochaetes (Lee and Hampson 1995). A MAb of the IgM subclass produced in mice has also been reported and, its specificity was demonstrated by the specific reaction with a 30 kDa cell envelope of *B. hyodysenteriae* (Lee and Hampson, 1996). Although, it was reported that other intestinal spirochaetes than *B. hyodysenteriae* have cell envelope proteins similar to that of *B. hyodysenteriae* but no reaction was observed with the MAb, this suggested that the epitopes might be different (Lee and Hampson, 1996).
Overall, serotyping of intestinal spirochaetes may be limited because of antigenic cross-reactivity and insufficient definition of antigens.

2.3. Molecular differentiation of intestinal spirochaetes.

The refinement of molecular typing methods has been useful for inter- and intra-specific differentiation of several micro-organisms with intestinal spirochaetes included among these. One of the most important aspects of differentiation of porcine intestinal spirochaetes is the separation of pathogenic from the non-pathogenic species. The early differentiation was based on the type of haemolysis on culture, being the strong β-haemolytic regarded as pathogenic and the weakly β-haemolytic as the non-pathogenic (Kinyon *et al,* 1977). However, as it emerged that WBHIS included pathogenic species, this characteristic was inadequate; hence, differentiation of intestinal spirochaetes has been assessed by various molecular methods including SDS-PAGE, DNA hybridisation

and sequencing of the 16S and 23S rRNA genes (Tables 1.3abcd). Most typing methods show a high degree of similarity within species and support the speciation. *B. hyodysenteriae* and *B. innocens* are the most closely related. *B. pilosicoli* and the other WBHIS are clearly distinct from *B. hyodysenteriae* and *B. innocens* group and from each other as established by phylogenetic studies.

2.3.1. Differentiation of intestinal spirochaetes by protein analysis (Table 1.3a).

Reference strains B204 of *B. hyodysenteriae* and B256 of *B. innocens* were first used in a study in which it was shown that these two species could be differentiated by separation of their proteins by gel electrophoresis (Joens and Marquez 1989). Proteins of molecular sizes 72, 53, 46 and 44 kDa were unique to *B. hyodysenteriae* whereas the proteins of molecular sizes 55, 47, 37, 27 and 19 kDa were observed only in *B. innocens*. These proteins distinguished the two species of intestinal spirochaetes (Joens and Marquez 1989). However, it was found that five proteins were common in both *B. hyodysenteriae* and *B. innocens*.

In a further study of differentiation of intestinal spirochaetes on the basis of electrophoretic analysis of proteins by Stanton *et al* (1992), it was found that reference strains of *B. hyodysenteriae* were ≥96 % similar to each other. Strains of *B. innocens* showed 79.0 % of protein similarity, while the similarity between the proteins of *B. hyodysenteriae* and those of *B. innocens* was at the level of 38.0 to 45.0 % (Stanton *et al,* 1992). Further, the similarity of the proteins of either *B. hyodysenteriae* or *B. innocens* was at the level between 23.0 to 31.0 % as compared to the proteins of *T. bryantii, T. succinifaciens* or *E. coli* (Stanton *et al,* 1991).

In another study using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) it was shown that the protein profiles of *B. hyodysenteriae* type strains

ATCC-27164 and ATCC-31212 were very similar to each other. *B. innocens* type strain ATCC-29796 was characterised by proteins of molecular sizes 41.1, 44.2, 59.4 and 72.4 kDa which differentiated this strain from *B. hyodysenteriae* (Ochai *et al,* 1997), and *B. pilosicoli* type strain ATCC-51139 was characterised by proteins with molecular sizes of 29.6, 33.3, 59.4 and 53.8 kDa which were different from the protein profiles of the type strains of *B. hyodyesnteriae* and *B. innocens.* The non-pathogenic human intestinal spirochaete *B. aalborgi* type strain NCTC 11492 had proteins of molecular sizes 29.6, 33.3 and 59.4 kDa in common with the type strain ATCC-51139 of *B. pilosicoli*, and the proteins of *B. aalborgi* molecular sizes 59.4 and 52.3 kDa were also in common with the type strain ATCC-29796 of *B. innocens* (Ochai *et al,* 1997).

Table 1.3a. Molecular comparisons between different species of intestinal spirochaetes (SDS-PAGE protein comparison)*.

	B. hyodysenteriae	*B. innocens*	*'B-S.' intermedia*	*'B-S.' murdochii*	*B. pilosicoli*	*B. Aalborgi*
B. hyodysenteriae	>96 %					
B. innocens	38-45 %	79 %				
'B-S.' intermedia	--	--	--			
'B-S.' murdochii	--	--	--	--		
B. pilosicoli	--	--	--	--	--	
B. aalborgi	--	--	--	--	--	--

"--" no comparisons done
* references cited on the text

2.3.2. Differentiaion of porcine intestinal spirochaetes by DNA comparisons (Table 1.3.b).

A study carried out by Miao *et al* (1978) showed DNA hybridisation at the level of 80.0 % between the strains B204 and A-1 of *B. hyodysenteriae*, and at the level of 90.0 % between the strains B256 and 4/71 of *B. innocens.* While the DNA hybridisation found between the strains of *B. hyodysenteriae* and *B. innocens* was only at the level of 28.0 % (Miao *et al,* 1978); therefore, it was concluded that these species were genetically distant groups of the genus *Brachyspira*. These genetic differences gave the

basis for the recognition of a different species then named *B. innocens* (Miao *et al,* 1978).

Low levels of DNA homology have also been reported between the non-pathogenic human intestinal spirochaete *Brachyspira aalborgi* type strain NCTC-11492 and the pathogenic pig intestinal spirochaetes *B. hyodysenteriae* type strain ATCC-27164, *B. innocens* type strain ATCC-29796, and *B. pilosicoli* type strain ATCC-51139 with homologies at the level of 22.0, 19.1 and 17.2%, respectively (Ochai *et al,* 1997), thus conferring distinction.

The demonstration that WBHIS associated with diarrhoea in pigs were genetically distant from other intestinal spirochaetes was confirmed by a genetic study on DNA cross-hybridisation in which *B. hyodysenteriae* reference strains B78 and B204, *B. innocens* reference strains B256 and 4/71, *T. succinifaciens* isolate 6091 (type strain ATCC-33096), and *'Brachyspira-Serpulina' jonesii* isolate 16 (type strain ATCC-49776) were used for DNA:DNA comparisons with 16 isolates of WBHIS from pigs. The results of that study showed that the labelled probe of one of the field isolates of WBHIS strongly hybridised with another 7 field isolates of WBHIS. No hybridisation was observed with the type strains of *B. hyodysenteriae, B. innocens* nor *T. succinifaciens*. But the field isolate of WBHIS used as probe showed low levels of cross-hybridisation with *'Brachyspira-Serpulina' jonesii* indicating that WBHIS associated with diarrhoea in pigs are more closely related to the human WBHIS than to *B. hyodysenteriae* or *B. innocens* (Ramanathan *et al,* 1993). However, not all the 16 field isolates of WBHIS showed cross-hybridisation with the DNA of the field isolate used as template.

Recently it has been reported that the DNA homology of *B. pilosicoli* isolated from three different hosts, humans, dogs and pigs was higher than 95.0 %, and the comparison with *B. hyodysenteriae* and *B. innocens* gave a homology less than 32.0 %

(Duhamel *et al*, 1995b). The DNA-DNA reassociation reported approximately 40.0 % of sequence homology between *B. hyodysenteriae* and *B. innocens* (Stanton *et al,* 1991).

Another comparative study on DNA sequence homology within species showed that *B. hyodysenteriae* strains B204 and A-1 had 93.0 % DNA homology, and *B. innocens* strains B256 and 4/71 had 87.0 % DNA homology. The DNA sequence homology between species showed that the homology between strains of *B. hyodysenteriae* and strains of *B. innocens* was at the level of 37.0 to 41.0 % (Stanton *et al,* 1991).

A further study by Stanton *et al* (1997) on the relative DNA association of intestinal spirochaetes found between 78.0 and 100% DNA homology within species (to heterologous reference strains), and 21.0 to 68.0 % DNA homology between five different species of *Brachypira* i.e. *B. hyodysenteriae* reference strains B78 and R1 (isolated from rhea), *B. innocens* reference strain B256; *B. pilosicoli* reference strains P43/6/78 and WES-B isolate, *'Brachyspira-Sepulina' intermedia* reference strains PWS/A and 2818.5, and *'Brachyspira-Sepulina' murdochii* strains 56-150 and 155-20 (Stanton *et al,* 1997).

The DNA reassociation values between the two reference strains PWS/A (type strain ATCC51140) and 2818.5 of *'Brachyspira-Serpulina' intermedia* and the reference strains B78 (type strain ATCC-27164) and R-1 of *B. hyodysenteriae*, B256 (type strain ATCC-29796) of *B. innocens* and P43/6/78 (type strain ATCC-51139) of *B. pilosicoli* were at the level of 68.0 %, 44.0 to 48.0 % and 30.0 %, respectively (Stanton *et al,* 1997). The DNA reassociation values together with the molecular content of G+C (guanine + cytosine) of 25.0 % in *Brachyspira-'Brachyspira-Serpulina' intermedia* compared to 27.0 % in *B. hyodysenteriae* gave the genetic basis for the designation of the new species *intermedia* (Stanton *et al*, 1997).

Reference strains 56-150 (type strain ATCC-51254) and 155-20 (ATCC51284) of *Brachyspira-'Brachyspira-Serpulina' murdochii* showed 28.0 to 37.0 %, 64.0 %, 29.0 %

and 37.0 to 45.0 % of DNA homology to the reference strains of *B. hyodysenteriae*, *B. innocens, B. pilosicoli* and *B. intermedia*, respectively. The values of DNA reassociation together with the G+C content of 27.0 % in *Brachyspira-'Brachyspira-Serpulina' murdochii* compared to the G+C content of 24.2 % of *B. hyodysenteriae* gave the genetic basis along for the designated new species *murdochii* (Stanton *et al*, 1997).

Differentiation between the type strains of *B. hyodysenteriae* and *B. innocens* and *Treponema succinifaciens* at genus level was clear since the DNA homology between them was at the low level of ≤5.0 % (Stanton *et al,* 1991). At the level of species Lee *et al* (1993b) reported high values on DNA hybridisation for various strains of *B. hyodysenteriae* in which the values were above 88.3 % between the type strain P18A and other three strains of *B. hyodysenteriae.* Also high levels of DNA hybridisation were found within human strains of *B. pilosicoli* in which the values were greater than 67.1 %. That study of Lee *et al* (1993b) also demonstrated that the human strain HRM7 of *B. pilosicoli* had low DNA hybridisation levels 18.6 % and 8.0 % with the reference strain B256 of *B. innocens* and with the reference strain B78 of *B. hyodysenteriae*, respectively, indicating that *B. pilosicoli* was distantly related to both *B. hyodysenteriae* and *B. innocens* (Lee *et al,* 1993b).

Table 1.3b. Genetic comparisons between different species of intestinal spirochaetes (DNA hybridisation comparison)*.

	B. hyodysenteriae	*B. innocens*	*'B-S.' intermedia*	*'B-S.' murdochii*	*B. Pilosicoli*	*B. aalborgi*
B. hyodysenteriae	78 % 80 % 93 %					
B. innocens	28 % 37-41 %	87 % 90 %				
'B-S.' intermedia	68 %	44-48 %	--			
'B-S.' murdochii	28-37 %	64 %	37-45 %	--		
B. pilosicoli	8 %	18.6 %	30 %	29 %	--	
B. aalborgi	--	--	--	--	--	--

"--" no comparisons done
* references cited on text

2.3.3. Differentiation of porcine intestinal spirochaetes by 16S rRNA gene comparisons (Table 1.3c).

Studies on the sequence comparison of 16S rRNA gene have revealed that three strains of *B. hyodysenteriae* showed a high level of similarity (99.8 to 99.9 %), and two strains of *B. innocens* were also highly similar showing similarity values of 99.2 %. The average similarity of the sequence of the 16S rRNA gene between strains of *B. hyodysenteriae* and strains of *B. innocens* was at the level of 99.4 %, and the level of similarity between *B. hyodysenteriae* and *B. innocens*, and other spirochaetes was lower, at level 76.5 % (Stanton *at al,* 1991). Interestingly, in that study by Stanton *et al* (1991) the similarity between *B. hyodysenteriae* and *B. innocens*, and *E. coli* was 74.2%, almost at the same level of similarity to other spirochaetes (76.5 %) (Stanton *et al,* 1991). Thus, confirming their distant relatedness.

On the basis of biochemical classification for 21 strains of intestinal spirochaetes based on indole production, hyppurate hydrolysis, α- and β- glucosidase, α-galactosidase activity, and sequencing of 16S rRNA gene using PCR, a phylogenetic classification for those spirochaetes was determined (Fellström *et al,* 1995). The intestinal spirochaetes represented the six biochemical groups (I, II, IIIa, IIIb, IIIc, and IV) described above (section 2.2). The segment U2 to U5 of the 16S rRNA gene contains the evolutionary variable regions V6, V7 and V8. The PCR generated a fragment of 532 nucleotides length in which the position of 6 nucleotides differentiated the 21 strains, and the nucleotides at positions 564, 603, 621 and 814 matched the biochemical classification for those strains. Groups I and II were not clearly differentiated by the PCR in that study but group IV was characterised by a T nucleotide at position 604 which was very different from the other strains, group IV includes *B. pilosicoli* (Fellström *et al,* 1995).

In another study on the 16S rRNA gene sequences the similarity matrix showed that the two type strains ATCC-27164 and ATCC-31212 of *B. hyodysenteriae* were 99.9% similar to each other, and the homology values between those type strains of *B.*

hyodysenteriae and the type strains ATCC-29796 of *B. innocens* and ATCC-51139 of *B. pilosicoli* were at the level of 99.5% and 98.6%, respectively (Ochai *et al,* 1997). Interestingly, the type strain NCTC-11492 of *B. aalborgi* showed 96.8% homology with the two type strains of *B. hyodysenteriae* (ATCC-27164 and ATCC-31212) and the type strain ATCC-29796 of *B. innocens*. A slightly lower 16S rRNA gene sequence homology (96.0 %) was found between that type strain of *B. aalborgi* and the type strain ATCC-51139 of *B. pilosicoli* (Ochai *et al,* 1997). Based on the results of the 16S rRNA gene sequence homology and DNA hybridisation between *B. aalborgi* and the pig intestinal spirochaetes *B. hyodysenteriae, B. innocens* and *B. pilosicoli* it was observed that the *B. aalborgi* was more closely related to *B. hyodysenteriae* and *B. innocens* than to *B. pilosicoli* (Ochai *et al,* 1997).

Analysis of a region of 283 bp on the 16S rRNA gene was carried out to identify 19 unclassified isolates of human intestinal spirochaetes (De Smet *et al,* 1998). Comparisons were done with the five known species of intestinal spirochaetes found in pigs i.e. *B. hyodysenteraie, B. innocens, B. pilosicoli, 'Brachyspira-Serpulina' intermedia* and *'Brachyspira-Serpulina' murdochii*. The results from that experiment showed that the 19 human isolates differed from *B. pilosicoli* by only 0.3 bp, and from the other species of *Brachyspira* by 10 to 18 bp. Based on those results and the biochemical reactions matching the characteristics of *B. pilosicoli*, the unclassified human isolates were identified as *B. pilosicoli* (De Smet *et al,* 1998). However, in that study it was reported that one of the human isolates did not match the characteristic sequence signature on the 16S rRNA gene of *B. pilosicoli* (Park *et al,* 1995; Ochai *et al,* 1997). The specific sequence signature of *B. pilosicoli* has been used to design primers for PCR to differentiate *B. pilosicoli* from other intestinal spirochaetes (Park *et al,* 1995).

Table 1.3c. Genetic comparisons between different species of intestinal spirochaetes (16S rRNA gene sequencing comparison)*.

	B. hyodysenteriae	*B. innocens*	*'B-S.' intermedia*	*'B-S.' murdochii*	*B. pilosicoli*	*B. aalborgi*
B. hyodysenteriae	99.8-99.9 %					
B. innocens	99.5 %	99.2 %				
'B-S.' intermedia	--	--	--			
'B-S.' murdochii	--	--	--	--		
B. pilosicoli	96.0 %	--	--	--	--	
B. aalborgi	--	--	--	--	96.9 %	--

"--" no comparisons done
* references cited on text

2.3.4. Differentiation of porcine intestinal spirochaetes by 23S rRNA gene comparisons (Table 1.3d).

Based on 23S rRNA gene comparisons have also been done. Amplification of the 23S rRNA gene by PCR and followed by digestion of the amplified product with restriction enzymes *Taq* I and *Alu* I clearly differentiated the pathogenic species *B. hyodysenteriae* and *B. pilosicoli* from the non-pathogenic species *B. innocens* and *B. murdochii* (Barcellos *et al,* 2000). The restriction patterns using *Taq* I characterised *B. hyodysenteriae* with a fragment of 134 bp, *B. pilosicoli* with a fragment of 166 bp, and *B. alvinipulli* with two unique fragments of 51 bp and 94 bp. Whereas the digestion products generated by *Alu* I characterised *B. pilosicoli* with a fragment of 309 bp and *B. murdochii* with a fragment 206 bp (Barcellos *et al,* 2000).

Sequencing comparisons of the 23S rRNA gene have also been done (Leser *et al,* 1997). Amplification of the 23S rRNA gene by PCR generated a sequence of approximately 2470 bp that differentiated the porcine intestinal spirochaetes. The sequence similarity between *B. hyodysenteriae* and *'Brachyspira-Serpulina' intermedia* was 99.2 % which corresponded to a difference of 20 nucleotides. The 23S rRNA gene sequence similarities between *B. hyodysenteriae* and *B. innocens* and *B. pilosicoli* were 98.7 % and 98.9 %, respectively. The 23S rRNA gene sequence similarity between

'Brachyspira-Serpulina' intermedia and *B. pilosicoli* was slightly lower (96.9 %) than the 99.2 % similarity between *'Brachyspira-Serpulina' intermedia* and *B. innocens* (Leser *et al,* 1997). The sequence analysis of the 23S rRNA gene showed that *B. pilosicoli* was more distantly related to *B. hyodysenteriae, 'Brachyspira-Serpulina' intermedia* and *B. innocens* than was *B. hyodysenteriae* to *B. innocens*. *B. hyodysenteriae* was more closely related to *'Brachyspira-Serpulina' intermedia* than to *B. innocens* (Leser *et al,* 1997).

Table 1.3d. Genetic comparisons between different species of intestinal spirochaetes (23S rRNA gene sequencing comparison)*.

	B. hyodysenteriae	*B. innocens*	*'B-S.' intermedia*	*'B-S.' murdochii*	*B. pilosicoli*	*B. aalborgi*
B. hyodysenteriae	--					
B. innocens	--	--				
'B-S.' intermedia	99.2 %	99.2 %	--			
'B-S.' murdochii	--	--	--	--		
B. pilosicoli	98.7 %	98.9 %	96.9 %	--	--	
B. aalborgi	--	--	--	--	--	--

"--" no comparisons done
* references cited on text

2.3.5. Differentiation of porcine intestinal spirochaetes by restriction enzyme analysis

On the basis of restriction fragment length polymorphism (RFLP) analysis of rRNA genes, 19 isolates of *B. hyodysenteriae* and 11 isolates of *B. innocens* were differentiated and compared with the reference strains of *B. hyodysenteriae, B. innocens* and *Treponema succinifaciens*, the comparison included serotypes from various countries (Li *et al,* 1992). The results of the RFLP showed that all the isolates and the reference strains of *B. hyodysenteriae* serotype 1 B78 and B234, serotype 2 B204 (US), serotype 3

B169 (Canada), serotype 4 A-1 (UK), serotype 5 B8044, serotype 6 B6933 and serotype 7 Ack 300/8 (The Netherlands) had the same rRNA restriction pattern and probed with photobiotin-labeled *B. hyodysenteriae* reference strain B204 rRNA. All *B. hyodysenteriae* isolates and reference strains were characterised by two specific bands of 3.6 and 4.3 kb when using the restriction enzyme *Sau*3A. Whereas the isolates and reference strains of *B. innocens* B256 and *T. succinifaciens* 6091 had a quite different and distinguishable pattern from that of *B. hyodysenteriae* (Li *et al,* 1992). Interestingly, a similar study by Sotiropoulos *et al,* (1994) using the restriction enzyme *Sau*3A compared many of the same reference strains and serotypes of *B. hyodysenteriae* and *B. innocens* as the previous study by Li *et al,* (1992). The latter study (Sotiropoulos *et al,* 1994) reported that differentiation between *B. hyodysenteriae* and *B. innocens* was characterised by a strong hybridisation of the 1.1 kb DNA probe specific for *B. hyodysenteriae* (Sotiropoulos *et al,* 1993) with a DNA fragment generated by the restriction enzyme *Sau*3A of molecular size 3.0 kb that was present in all the strains of *B. hyodysenteriae* tested. This 3.0 kb fragment generated by *Sau*3A was proposed to be used as species specific since it was observed in all the reference strains of *B. hyodysenteriae* indicating that it is highly conserved. No hybridisation of the 1.1 kb DNA probe specific for *B. hyodysenteriae* was observed with the digestion products of the DNA of *B. innocens* B256. Digestion products of the DNA of *B. innocens* using the restriction enzyme *Asp*700 did not hybridise with the 1.1 kb DNA specific for *B. hyodysenteriae* either, which confirmed that *B. hyodysenteriae* and *B. innocens* are different to each other (Sotiropoulos *et al,* 1994). The use of a 0.75 kb DNA probe also specific for *B. hyodysenteriae* (Sotiropoulos *et al,* 1993) together with the 1.1 kb DNA probe proved to be very useful for epidemiological differentiation of *B. hyodysenteriae* isolates when applied simultaneously (Sotiropoulos *et al,* 1994).

2.3.6. Differentiation of porcine intestinal spirochaetes by haemolysin and flagellar genes

In another study by ter Huurne *et al,* (1992a), the differentiation between field isolates of *B. hyodysenteriae* and *B. innocens* was conducted by comparing their restriction enzyme fingerprints with those of the reference strains (including many of the same reference strains as mentioned above). The extent of hybridisation with two *B. hyodysenteriae* DNA fragments used as probes a 2.2 kb region coding for a flagellar protein (Fla probe), and a 1.4 kb DNA sequence containing the *tly* gene coding for haemolysin (Tly probe). The results showed that the 43 field isolates of *B. hyodysenteriae* exhibited different DNA patterns, most of them were included in restriction enzyme patterns I and II, and their patterns were clearly distinguishable from those of *B. innocens*. In comparison, the DNA patterns observed on the 10 field isolates of the non-pathogenic *B. innocens* were more diverse and only two isolates showed the same DNA pattern. The differentiation between the two species was more clear at the hybridisation level since DNA fragments of 4 and 14 kb from restriction pattern I, and DNA fragments of 4 and 4.2 kb from restriction pattern II of *B. hyodysenteriae* isolates strongly hybridised with the Fla probe. Hybridisation of a DNA fragment of 6.5 kb from all the field and reference strains of *B. hyodysenteriae* strongly hybridised with the Tly probe. In contrast, various DNA fragments of 3.1, 3.7, 6.5, 7.2, 8.3, 8.5 and 15 kb from the field isolates and the reference strain of *B. innocens* hybridised weakly with the Fla probe and no traces hybridisation of *B. innocens* were detected with the Tly probe (ter Huurne *et al,* 1992a).

By restriction fragment length polymorphism (RFLP) of the periplasmic flagella *flaA*$_1$ gene it was possible to distinguish between *Serpulina* and *Brachyspira* species. Moreover, the fingerprints of *B. hyodysenteriae, B. innocens, B. pilosicoli* and the provisionally designated '*Brachyspira-Sepulina' murdochii* and '*Brachyspira-Serpulina' intermedia* were species characteristic, therefore this method of differentiation proved to be useful (Fisher *et al,* 1997).

2.3.7. Differentiation of porcine intestinal spirochaetes by multiplocus enzyme electrophoresis (MEE)

The genetic relatedness between strains of *B. hyodysenteriae* and strains of *B. pilosicoli* has also been determined by comparative studies using MEE. On a phylogenetic tree derived from MEE data the species *B. hyodysenteriae* clustered together into group A. *B. pilosicoli* formed a cluster together, indicating that *B. pilosicoli* isolates belonged to the same genetic group (designated as group C), and were genetically distant from the strains of group A (*B. hyodysenteriae*) (Lee *et al,* 1993a; Lee *et al,* 1993b), in conjunction with DNA hybridisation and other methods.
Another study on MEE separated isolates into three different groups A, B and C, group A corresponded to isolates of *B. hyodysenteriae, B. intermedia* and *B. innocens*, group B and C grouped pathogenic isolates of weakly β-haemolytic intestinal spirochaetes. Group C had a genetic distance of 0.79 from the other two groups, and was shown to be a group of intestinal spirochaetes different from *B. hyodysenteriae* and *B. innocens* (Lee *et al*, 1993a).

A further classification of intestinal spirochaetes on the basis of MEE separated them into seven groups (I to VII). *B. hyodysenteriae* represents group I, *'Brachyspira-Serpulina' intermedia* group II, *B. innocens* group III, *'Brachyspira-Serpulina' murdochii* group V, *B. pilosicoli* group VI and *B. aalborgi* group VII (Lee *et al,* 1993a; Lee *et al,* 1993c; Stanton *et al,* 1996).

Another genetic parameter to differentiate spirochaetes is by comparing the percentage of molecular content of guanine and cytosine. The G+C content of *B. hyodysenteriae* and *B. innocens* has been reported to be at the level of 26 % (Kinyon and Harris 1979). Similar values of G+C content of *B. hyodysenteriae* and *B. innocens* values 25.8 and 25.6 %, respectively have been reported (Ochai *et al,* 1997), and the G+C values of

B. pilosicoli and *B. aalborgi* have been observed at the level of 24.9 and 27.1 %, respectively (Ochai *et al,* 1997). Whereas the G+C contents of the genus *Treponema* species *T. bryantii* and *T. pallidum* have been reported to be at the level of 36.0 % and 53.0 % which are significantly different from those of the genus *Brachyspira* that range from 24.0 to 27.0 % (Stanton and Canale-Parola 1980; Miao and Fieldsteel 1978).

2.4. *Brachyspira pilosicoli*

Some of the characteristics of *Brachyspira pilosicoli* have been mention previously through this manuscript, particularly in section 2.2. This section provides a more detailed description of this micro-organism.

Colonies of *B. pilosicoli* produce a weak β-haemolysis on BA plates, and the cells are sufficiently distinct to differentiate them from *B. hyodysenteriae* by electronic microscopy. *B. pilosicoli* cells are 4 to 12 μm long and 0.2 to 0.3 μm wide (Trott *et al,* 1996c). *B. pilosicoli* isolated from both pigs and humans are more similar morphologically to *B. aalborgi* since both species have between 4 and 6 periplasmic flagella at each end (Lee *et al,* 1993a; Lee *et al,* 1993b), and similar diameter but *B. aalborgi* is shorter 1.7 to 6.0 μm, and less coiled which makes them look comma-like (Hovind-Hougen *et al,* 1982). *B. pilosicoli* grows well on trypticase soy blood agar and brain heart infusion supplemented with foetal calf or rabbit serum at 37 to 42 °C, whereas the growth of *B. hyodysenteriae* and *B. innocens* is more limited at higher temperatures, and *B. aalborgi* does not grow at 42 °C (Hovind-Hougen *et al,* 1982). *B. pilosicoli* does not grow at 32 °C or 45 °C. The doubling time of *B. pilosicoli* is between 1 to 2 hrs in BHI broth at 38 °C (Trott *et al,* 1996c), which is shorter than that of *B. hyodysenteriae* (3-5 hrs).

The growth characteristics of *B. pilosicoli* are slightly different from those of *B. hyodysenteriae* and *B. innocens.* For instance, *B. pilosicoli* has an oxygen tolerance of

7.0 % as compared to the 5.0 % tolerance of *B. hyodysenteriae* (Trott *et al,* 1996c). The atmospheric pH for the optimum growth of *B. pilosicoli* varies from 5.6 to 8.0 as compared to that between 6.0 to 8.0 of *B. hyodysenteriae,* or 6.5 to 8.0 of *B. innocens*. *B. pilosicoli* is the only known species of intestinal spirochaetes that uses D-ribose as source of energy together with soluble sugars such as pyruvate, D-glucose, D-fructose, D-sucrose, D-trehalose, D-glucosamine, D-mannose, maltose, L-fucose and D-cellobiose (Trott *et al,* 1996c). *B. pilosicoli* does not grow in the presence of the substrates L-arabinose, D-fucose, D-raffinose, D-melibiose, L-rhamnose, D-xylose, galactosamine, N-acetylgalactosamine, cellulose, esculin, soluble starch, pectin, mucin, glycogen, glucuronic acid, polygalacturonic acid, glutathione, glycerol, mannitol, acetate and lactate (Trott *et al,* 1996c). Certain human strains of *B. pilosicoli* are characterised by using D-xylose as a substrate for growing which differentiates them from porcine isolates (Trott *et al,* 1996b). *B. pilosicoli* is resistant to several antibiotics that include colistin, polymyxin B, spectinomycin and vancomycin, some strains of *B. pilosicoli* are also resistant to ampicillin and streptomycin (Trott *et al,* 1996b). This natural resistance of intestinal spirochaetes to these antibiotics has been considered for developing selective culture media to improve their isolation (in pure culture) from faecal samples (Kunkle and Kynion 1988; Calderaro *et al,* 2001).

3. Pathology.

B. pilosicoli is pathogenic for pigs, other domestic animals such as dogs, chickens and also humans, and produces consistent intestinal disorders in these hosts. The disease in pigs was firstly named spirochaetal diarrhoea (Taylor *et al,* 1980), and the disease in humans was known as intestinal spirochaetosis (Harland and Lee 1967). The term that refers to the pathological condition in pigs changed in recent years, the name given is porcine colonic spirochaetosis (PCS) (Duhamel *et al,* 1995a) or porcine intestinal spirochaetosis (PIS) (Trott *et al,* 1996c).

Infection caused by *B. pilosicoli* is localised in the large intestine, the colon being the most affected portion. The mortality is low but the morbidity may be as high as 75.0 % (Taylor, 1995). *B. pilosicoli* infection in pigs is characterised by a transient greyish catarrhal diarrhoea occasionally containing blood. The disease is commonly seen in weaned pigs of 6 to 12 weeks of age (Taylor *et al,* 1980; Duhamel *et al*, 1995a; Fellström and Gunnarsson, 1995), which coincides with the post-weaning period, and as a consequence of diarrhoea, pigs show poor performance and reduced growth rates. However, pigs of any age are susceptible and may be affected since the disease has also been seen in finishing and adult pigs. The incubation period of the disease is between 5 and 20 days during which a slight fever (40 °C) may develop, recovery may occur within 7 and 10 days after infection and the mortality rate is almost zero (Taylor, 1995).

The macroscopic lesions in the pig as result of infection with *B. pilosicoli* are observed in the colon and caecum which their contents may be grey and watery. The lesions include oedematous serosal surface, enlarged mesenteric lymph nodes, areas of multifocal erosion and necrosis, congestion and thickening of the colonic mucosa with adherent fibrinous exudate (Girard *et al* 1989; Duhamel *et al,* 1995a; Trott *et al,* 1996a; Thomson *et al* 1997, Thomson *et al,* 1998). However macroscopic lesions are not always visible. In chronic cases the haemorragic spots on the mucosa are covered with fibrin, necrotic material and digesta observed as conical adhesions. The pathological findings of PCS are never as severe as swine dysentery (Duhamel *et al,* 1995a; Trott *et al* 1996a; Thomson *et al* 1997).

The histopathological changes after infection with *B. pilosicoli* have been described as multifocal ulcerative colitis with large numbers of invasive bacteria attached by one end to the surface of the intestinal epithelium (Taylor *et al,* 1980; Girard *et al,* 1989; Duhamel *et al,* 1995a; Trott *et al,* 1996a; Thomson *et al,* 1997; Trivett-Moore *et al,* 1998, forming a false brush border which is considered as pathognomonic sign of the disease. The lesions are mainly observed on the mucosa and submucosa but the muscularis may be also affected (Thomson *et al,* 1996; Trott *et al,* 1996a). An increase

in the number of goblet cells, and the numbers and types of inflammatory cells including mononuclear and polymorphonuclear cells has also been observed (Neef *et al,* 1994; Hampson *et al,* 2000). And dilatation of intestinal crypts containing high amounts of mucus and debris that could give origin to crypt abscesses (Girard *et al,* 1995; Trott *et al,*1996a; Thomson *et al,* 1997). The presence of *Ballantidium coli* cells in large quantities on the intestinal epithelium in conjunction with the infection of *B. pilosicoli* has been reported (Taylor *et al,* 1980; Spearman *et al,* 1988; Trott *et al,* 1996a; Hampson *et al,* 2000). It was initially considered that the diarrhoea seen in the infection by *B. pilosicoli* was as result of the attachment of spirochaetes to the intestinal epithelium resulting in a decreased absorption of epithelial cells in the colon (Schmall *et al,* 1983). Though, relatively little is known about specific virulence mechanisms by which *B. pilosicoli* (or other intestinal spirochaete) cause infection, there has been some advancement in our understanding of bacterial factors as well as host responses.

3.1. Virulence factors of intestinal spirochaetes

Virulence factors of intestinal spirochaetes have been described and include: motility, invasion and adhesion which play a role in colonization. Other factors include toxins such as cytotoxins, haemolysins, enterotoxins, endotoxins and hydrolytic enzymes (e.g. proteases and hyaluronidase), which act at molecular level. Most studies on virulence factors have been elucidated using *B. hyodysenteriae* as a model for *in vivo* and *in vitro* experiments, although some of these features may also apply to infection with *B. pilosicoli* and other intestinal spirochaetes.

3.2. Motility.

Mucus eliminates unattached micro-organisms efficiently from the gastrointestinal tract, and contrary to other enteric pathogens, *B. hyodysenteriae* and *B. pilosicoli* are highly motile in the presence of mucus (Kennedy *et al,* 1988; Witters and Duhamel, 1999). Therefore, motility is recognised as an important virulence factor of intestinal spirochaetes allowing them to escape the physical effect of mucus (Guerry *et al,* 1991). Colonisation of the mucosa by *B. hyodysenteriae* and *B. pilosicoli* can be explained by the fact that these micro-organisms are chemotactic to mucin, and use the components D-glucose, D-galactose, N-acetyl-D-glucosamine and D-glucosamine (which constitute the pig colonic mucin glycoproteins) as substrate for growing (Zhang *et al,* 2000). Another study confirmed that mucin induced strong chemotaxis to *B. hyodysenteriae* as did blood, fucose and serine. Though, maximal chemotaxis was dependent upon the physiological status of the spirochaete such as culture on mid to late logarithmic phase, cell concentration (1 X 10^9 cells/ml) and time of incubation (1-2 hrs) (Kennedy and Yancey, 1996).

The motility of intestinal spirochaetes is provided by periplasmic flagella. These structures are found in a protoplasmic cylinder contained within the cell envelope (Harris and Lysons, 1992). The periplasmic flagella of most intestinal spirochaetes are complex structures made up of several different polypeptides (Charon *et al*, 1992). For instance, the periplasmic flagella of *B. hyodysenteriae* have been characterised and contain three antigenically related polypeptides designated as $FlaA_1$ (42 kDa), $FlaB_1$ (38 kDa) and $FlaB_2$ (31 kDa) (Koopman *et al,* 1992; Gabe *et al,* 1995). The periplasmic flagella of intestinal ispirochaetes differ from the flagella composition of other bacteria that are composed of a single flagella protein called flagellin (Macnab, 1987). The flagellar proteins of intestinal spirochaetes share immunological features to each other and also with the flagellar proteins of other spirochaetes (Charon *et al,* 1992).

Genetic manipulation of *B. hyodysenteriae* produced $FlaA_1$ and $FlaB_1$ mutant strains and demonstrated that flagellar mutant strains lack pathogenicity. This indicated that periplasmic flagella are an important mechanism of virulence, and it seems that $FlaA_1$ and $FlaB_1$ are compensatory to each other since only dual mutants affect the *in vitro* motility and consequently the ability of *B. hyodysenteriae* to colonise the intestine of pigs and cause disease (Rosey *et al,* 1995; Rosey *et al,* 1996).

Structural and functional studies have been favoured after the cloning of genes that code for the $FlaA_1$, $FlaB_1$ and $FlaB_2$ proteins. The gene that codes for $FlaA_1$ generates a protein of 44 kDa containing 320 amino acid residues (Koopman *et al,* 1992; Koopman *et al,* 1993). While the gene encoding the $FlaB_1$ protein contains 290 amino acid residues (Gabe *et al,* 1995).

Flagellar components may be important immunogens. For instance, in one study five polypeptides were identified as components of the axial filaments of *B. hyodysenteriae* strain P18A and were described as AF_1 (43.8 kDa), AF_2 (38.0 kDa), AF_3 (34.8 kDa), AF_4 (32.8 kDa) and AF_5 (29.4 kDa), of which the polypeptide AF_5 was found to be present in all serotypes of *B. hyodysenteriae.* In that study, the sera of convalescent pigs recognised this polypeptide suggesting that axial filament components might play a role as a major immunodominant antigens (Kent *et al,* 1989). It is unclear which of the proteins will be a protective antigen since the serum of vaccinated pigs recognised the $FlaB_1$ protein in immunoblots, the vaccinated pigs were still susceptible to experimental challenge, however (Gabe *et al,* 1995). Although flagella are undoubtedly important virulence factors, the pathogenesis of SD may develop in conjunction with other virulence factors which also play significant roles in infection (Gabe *et al,* 1995).

3.4. Adhesion.

The adhesion of intestinal spirochaetes to epithelial cells has been reported as a potential virulence factor (Binek and Szyenkiewicz, 1986). Adhesion of *B. hyodysenteriae* to epithelial cells was demonstrated in a study *in vitro* where the adhesion was optimal in a neutral pH, and the adhesion was reduced at a pH >8.0. In addition that study showed that the hyperimmune and convalescent sera reduced the adhesion of *B. hyodysenteriae* by as much as 80 % (Bowden, *et al,* 1989). Moreover, in another study the attachment of individual cells of *B. hyodysenteriae* to the intestinal luminal surface of one-day old chickens has been observed but there was no evidence of invasive attachment to epithelial cells, a characteristic of *B. pilosicoli* (Trott *et al,* 1995). However, it has been proposed that chemotaxis to mucus and high speed motility of *B. hyodysenteriae* facilitate colonisation and penetration of the mucosa but adhesion of *B. hyodysenteriae* to the mucosa is not necessary to cause disease (Kennedy *et al,* 1988; Milner and Sellwood, 1994). Unlike *B. hyodysenteriae*, adhesion of *B. pilosicoli* to epithelial cells is believed to be necessary to cause disease. A study showed the characteristic attachment of *B. pilosicoli* (isolates from three host species: pigs, dogs and humans) to epithelial cells after inoculation into chickens (Trott *et al,* 1995; Muniappa *et al,* 1996). Adhesion was followed by ultrastructural observations and suggested the presence of adhesin-receptor interactions (Muniappa *et al,* 1996). As yet, no specific adhesin has been identified in *B. pilosicoli* or other members of the genus. However, adhesion of *B. pilosicoli* to epithelial cells after infection in pigs is not always seen on histopathological examinations (Thomson *et al,* 1997).

In experimental studies *B. innocens* has not been able to cause disease (Trott *et al,* 1995), and does not adhere to the intestinal epithelium. However, in a challenge study the strains 22/7, P280/1 and 14/5 of *B. innocens* showed to be pathogenic in gnotobiotic pigs (Neef *et al,* 1994). Although adhesins may be important, at least for some of the intestinal spirochaetes, the significance of this is not fully elucidated.

3.5. Invasion

The significance of epithelial invasion by intestinal spirochaetes varies. The ability of *B. hyodysenteriae* to invade epithelial cells is controversial since it has been reported that *B. hyodysenteriae* has been seen on the basal membrane of epithelial cells after diminishing cellular junctions either by enzymatic or cytokine activity of affected cells (ter Huurne and Gaastra, 1995). Invasiveness of *B. hyodysenteriae* has been regarded as a not essential feature to produce pathological lesions in the intestinal epithelium (Glock *et al,* 1980). However, invasive colonisation is a known characteristic of *B. pilosicoli,* and it has been observed within goblet cells, within the cytoplasm of enterocytes, between enterocytes and in the lamina propria (Duhamel *et al,* 1995a; Thomson *et al,* 1996).

The invasive mechanism of *B. pilosicoli* may be unique and certainly differs from other bacteria (Hartland *et al,* 1998), since the DNA sequences of the genes *inv, ail, yadA* and *eae* from *E. coli, Shigella flexneri* and *Yersinia enterocolitica* known to be involved in attachment and invasion did not hybridise with DNA of *B. pilosicoli* when used as probes (Hartland *et al,* 1998). However, no further studies have been conducted.

It has been suggested that the invasive colonisation of intestinal spirochaetes to the intestinal epithelium is a result of the interaction between a receptor-adhesin (ligand) on the surface host cells and the intestinal spirochaetes (Duhamel *et al,* 1993). However, this has yet to be specified. That interaction may cause a disruption of cell to cell communication resulting in the absence of cell chemotactic cytokine production and consequently decreasing the inflammatory response as a consequence (Muniappa *et al*, 1997).

3.6. Haemolysin/cytotoxin

Haemolytic activity is a characteristic of intestinal spirochaetes and some progress has been made in characterising the haemolysin determinant(s) responsible.

Haemolysin is perhaps the most important virulence factor of intestinal spirochaetes, particularly for *B. hyodysenteriae*. The amount of haemolysin produced is considered to be important among pathogenic strains. It has been suggested that strains of *B. hyodysenteriae* producing higher amounts of haemolysin were more virulent than those producing lower levels (Karen *et al,* 1984). Haemolysin showed no significant differences in lytic activity to erythrocytes of dog, cattle and sheep, but the erythrocytes of pigs and humans were less sensitive to haemolysins of *B. hyodysenteriae* (ter Huurne *et al,* 1994). The haemolysin itself is cytotoxic to different cell lines under laboratory conditions (Saheb *et al,* 1981). The cytotoxicity of purified haemolysin of *B. hyodysenteriae* on epithelial cells of ligated loops of colon and caecum of pigs was observed within 30 minutes of exposure (Lysons *et al,* 1991). Therefore, haemolysis is due to factors which are closely involved in disease processes, and the lytic effect is a consequence of an osmotic mechanism that causes lysis via pore formation, as demonstrated using purified haemolysin with sheep red blood cells (Hyatt *et al,* 1994). The haemolysin of *B. hyodysenteriae* is active at a temperature range between 27 and 42 °C and a pH range between 3.0 and 9.0 (Hyatt *et al,* 1994).

To date three haemolysin genes of *B. hyodysenteriae* have been characterised and designated as *tlyA, tlyB* and *tlyC* (Muir *et al,* 1992; ter Huurne *et al,* 1992b and 1994). The *tlyA* gene encodes a haemolysin protein of molecular size 26.4 kDa which is responsible for the strong β-haemolysis therefore, is only present in *B. hyodysenteriae*. The *tlyB* and *tlyC* genes in *B. hyodysenteriae* encode haemolysin proteins of 30.8 and 93.3 kDa, respectively. *B. innocens* may have genes related to the *tlyB* and *tlyC* genes of *B. hyodysenteriae* (ter Huurne *et al,* 1994). Immune serum from infected pigs did not recognise the haemolysin TlyA, suggesting that it might not be a strong immunogen. Interestingly, no homology has been found between the haemolysin of *B. hyodysenteriae* and the haemolysins of other bacteria (Muir *et al,* 1992).

Among the haemolysins, TlyA has been shown to have a significant role in pathogenesis. Although, a mutant strain missing the *tlyA* gene (*tlyA*$^-$) showed reduced

virulence since the pigs inoculated did not develop the typical clinical signs of SD, and the shedding was of much shorter duration than that of the pigs inoculated with the wild type strains (Hyatt *et al,* 1994). Although pigs inoculated with the *tlyA*⁻ mutant strain still developed mild clinical signs as compared to those pigs inoculated with the wild type strains B204 and C5. Moreover, pigs previously inoculated with the *tlyA*⁻ mutant strain developed partial protection when challenge with the wild type strains B204 and C5 since no lesions were developed (Hyatt *et al,* 1994). These studies indicate that the haemolysin plays a main role in the pathogenesis of *B. hyodysenteriae* (Hyatt *et al,* 1994) but other factors are believed to contribute to the pathogenesis.

The N-terminal amino acid sequence of the purified β-haemolytic toxin of *B. hyodysenteriae* was obtained and used to compare it with the previously known genes *tlyA, tlyB* and *tlyC* coding for the haemolysins of *B. hyodysenteriae*. Due to no homology with none of the known genes coding for haemolysin, the sequence of the N-terminal was cloned, designated as *hlyA* and expressed in *E. coli*. Recombinant strains of *E. coli* produced β-haemolysis on BA plates, and showed identical protease and lipase activities as the native purified β-haemolysin of *B. hyodysenteriae*. By hybridisation the gene *hlyA* was demonstrated to be present in *B. hyodysenteriae* and *B. intermedia* but absent in *B. innocens, B. pilosicoli* and *B. murdochii*, and its location on the chromosome was different from that of the known genes *tlyA, tlyB* and *tlyC* (Hsu *et al,* 2001).

3.7. Immunogens

Although some molecular structures acting as antigens in *B. hyodysenteriae* infection have been recognised using immune sera of pigs (recovered from SD), and polyclonal antibodies and MAbs against *B. hyodysenteriae* and *B. pilosicoli* have been produced, the immune response to these species or other intestinal spirochaetes is not well understood.

A protein of 16 kDa identified in strain B204 of *B. hyodysenteriae* but not in strain B256 of *B. innocens* by sera of convalescent pigs suggested that this protein might be involved in the immune response since sera of pigs recovered from SD recognised that antigen in immunoblots (Joens and Marquez 1986). The 16 kDa protein was also identified in the reference strains B78 and S80/5, and field isolates of *B. hyodysenteriae*. Antisera raised in rabbit recognised the 16 kDa protein in *B. hyodysenteriae* but not in any of the other intestinal spirochaetes, suggesting that the immune response to that protein of *B. hyodysenteriae* may be species-specific. However, variation in reaction between the different isolates of *B. hyodysenteriae* were observed possibly as a result of heterogeneity among serotypes (Thom and Taylor, 1993).

A predominant band of 16 kDa has also been found in *B. pilosicoli* as a component of the lipo-oligosaccharides extract; however, antisera to *B. pilosicoli* cells raised in rabbits did not recognise that 16 kDa component in immunoblots. The antisera recognised components at the range of 20-30 kDa of *B. pilosicoli* lipo-oligosaccharides extracts, and no cross-reaction was observed with the extracts from other species of *Brachyspira* (Lee and Hampson, 1999). This suggests that the antigenic determinants of *B. hyodysenteriae* and *B. pilosicoli* may be different.

Polypeptide antigens from the cell envelope of 11 strains including the reference strains B78, B204 and B169 of *B. hyodysenteriae* were obtained and immunoblotted with antisera to the whole cells of *B. hyodysenteriae* raised in pigs and rabbits. It was shown that *B. hyodysenteriae* had conserved antigens in the range 30 to 36 KDa. The pig antiserum recognised those antigens (associated with the cell envelope) but also the antigens of molecular size 29 to 45 kDa (Chatfield *et al,* 1988).

Despite identification of immunogens among the pathogenic *Brachyspira* species, the importance in virulence remains to be defined.

3.8. Experimental reproduction of *Brachyspira pilosicoli* infection

Although virulence of intestinal spirochaetes is conferred by multiple components, most of them have yet to be defined. Despite this, pathogenicity has been clearly demonstrated in experimental challenge studies which have confirmed that the clinical signs and histological changes produced by *B. pilosicoli* and *B. hyodysenteriae* in the large intestine are distinct (Taylor *et al,* 1980). Experimental challenge has reproduced the clinical signs of PCS in conventional pigs (Taylor *et al*, 1980; Thomson *et al*, 1996; Trott *et al,* 1996a), gnotobiotic pigs (Neef *et al,* 1994), and chickens (Muniappa *et al,* 1996; Trott *et al,* 1995).

In conventional pigs *B. pilosicoli* produced clinical signs between 4 and 8 days after experimental infection with isolates recovered from field outbreaks (Thomson *et al,* 1997). Unexpectedly, the characteristic attachment of *B. pilosicoli* to epithelial cells of the colon was not observed in histological preparations at 14 days post inoculation (Thomson *et al,* 1997). However, the significance of this was unclear.

Inoculation of gnotobiotic pigs with *B. pilosicoli* type strain P43/6/78 and the field strain 2/7 also reproduced clinical signs and lesions consistent with PCS (Neef *et al,* 1994). However, the lesions were observed in the caecum rather than in the colon, and not all the pigs inoculated with those strains of *B. pilosicoli* developed infection, as two of the strains of *B. pilosicoli* were not pathogenic in gnotobiotic nor conventional pigs (Neef *et al,* 1994). In the same study, it was also shown that the severity of clinical signs of gnotobiotic pigs fed with a liquid diet (based on milk) inoculated with *B. hyodysenteriae* were relatively mild. Those results suggested that factors included in the diet (cereal based) and the normal flora might play a role in the exacerbation the pathogenicity of *B. hyodysenteriae* (Neef *et al,* 1994).

Similarity in pathogenicity between isolates from humans, dogs and pigs has been demonstrated when inoculated into one-day-old specific pathogen free chickens. The isolates of human, canine and porcine origin produced the infection in chickens consistent with that found in naturally infected cases of colonic spirochaetosis in pigs and humans (Trott *et al,* 1995; Muniappa *et al,* 1996). Experimental studies have also shown that isolates of *B. pilosicoli* from humans were able to cause significant pathological changes in the large intestine of newly weaned pigs (Trott *et al,* 1996a), showing that this organism has the potential to cross species barriers. Isolates of *B. pilosicoli* from humans and pigs were also inoculated into newly weaned pigs which developed the characteristic infection, although the infection rate with the porcine isolate was higher (33.3 %, 4/12) than the rate with the human isolate (16.6 %, 2/12) (Trott *et al,* 1996a). Moreover, the porcine isolate produced the characteristic clinical signs between 2 and 10 days after inoculation, whereas the human isolate produced the clinical signs of PCS only after 11 days of inoculation.

These studies have demonstrated that *B. pilosicoli* might be able to cross the species barrier, however, differences in the level of virulence were observed between isolates (Muniappa *et al,* 1996).

B. pilosicoli isolated from pigs and chickens and *S. jonesii* isolated from humans were inoculated into two genetic lines of mice, C3H/HeJ and C3H/HeOuJ to study the pathogenesis of infection (Sacco *et al,* 1997). *B. pilosicoli* and *S. jonesii* were recovered on culture from all the inoculated mice after 4 and 20 days of challenge. At a histological level, the avian and porcine isolates were attached end-on to the apical surface of enterocytes in the caecum but the human isolate *S. jonesii* was not seen attached to the intestinal epithelial cells even 30 days after challenge (Sacco *et al,* 1997). The reproduction of *B. pilosicoli* infection in mice with isolates from three different hosts suggests that this micro-organism has a great capacity to adapt, colonise and cause infection in wide range of hosts. Actual transmission between different hosts and potential as zoonotic pathogen remains to be resolved.

Although comparative studies on susceptibility and pathogenesis in different genetic lines or breeds of pigs with either *B. hyodysenteriae* or *B. pilosicoli* have not been done, studies in mice have demonstrated that certain genetic lines are more susceptible to infection with *B. hyodysenteriae* than others. For instance, the genetic lines DBA/1J and BALB/cByJ required a higher infective dose ID_{50} than the genetic lines C3H/HeJ and C3H/HeN. Also those genetic lines that were more resistant to infection with *B. hyodysenteriae* showed quicker resolution of caecal lesions (Nibbelink and Wannemuehler, 1991). Comparing the two genetic lines C3H/HeJ and C3H/HeN, it was also observed that C3H/HeN was more susceptible than C3H/HeJ. Cecal lesions developed in C3H/HeN with as low as 100 cells of *B. hyodysenteriae* whereas CH3/HeJ did not develop lesion with that infective dose. When exposed to the same higher infective dose (1×10^5) C3H/HeJ developed fewer caecal lesions than CH3/HeN, and the number of *B. hyodysenteriae* cells excreted after infection with a dose of 1×10^6 was also significantly less. A possible explanation for the quicker clearance of lesions in the caecum of mice C3H/HeJ might be because this genetic line produces less tumour necrosis factor and interleukin-1 and therefore the inflammatory response is less dramatic, thus acute damage is lower, allowing quicker recovery and less predisposition to chronicity.

4. Genetic variation within the species *Brachyspira pilosicoli*

By all methods (REA-DNA, RFLP, MEE, pulse field gel electrophoresis-PFGE, 16S rRNA sequencing and random amplification of polyphormic DNA-RAPD), *B. hyodysenteriae* and *B. innocens* show to be more closely related, and the remaining WBHIS are clearly distinct from the *B. hyodysenteriae* and *B. innocens* and from each other establishing clear phylogeny (section 2.3). Moreover, genetic variation within the species *B. pilosicoli* has been observed at different levels of genetic analysis (see Chapter 4).

5. Epidemiological features.

Since *B. pilosicoli* is able to infect a wide range of hosts and has the potential to cross species barriers, its epidemiology is complex and of interest. It is already apparent that the epidemiology of PCS is complex, however, there are some aspects that remain to be studied in order to improve the understanding of this infection and develop better control and prevention strategies. On the other hand, the knowledge concerning the closely related *B. hyodysenteriae* is more vast and some epidemiological aspects may also apply to infection with *B. pilosicoli*.

5.1. Prevalence/incidence of porcine colonic spirochaetosis

One of the most common sources of information for epidemiological surveys on animal diseases is the abattoir. More epidemiological studies have been carried out on *B. hyodysenteriae* than on *B. pilosicoli*.

A serological survey was carried out to estimate the prevalence of *B. hyodysenteriae* in West and South Australia in which the prevalence found was 33.0 % and 36.0 %, respectively (Mhoma *et al*, 1982). Earlier, serological studies had suggested that more than one serotype might be present in a single herd. Sera from single herds showed cross-reactivity between the serotypes A, B, and C (Mhoma *et al*, 1979).

A survey of enteropathogens in 85 pig farms in the UK was carried out from 1992 to 1996. The figures of that study showed that *B. pilosicoli* infection was present as a single pathogen in 25.0 % of the cases, and in a mixed infection together with *Yersinia pseudotuberculosis*, *Salmonella typhimurium*, *Lawsonia intracellularis* and *B. hyodysenteriae* in 27.0 % of the outbreaks (Thomson *et al*, 1998). The prevalence estimated from that study of clinical outbreaks was between 5.0 and 15.0 %, and the mortality less than 1.0 %. Co-infection with *B. pilosicoli* and other pathogens such as

Lawsonia intracellularis has also been found in a study in Denmark in which 2.5 % (18/720) of the pigs with clinical signs of diarrhoea were simultaneously infected with both bacterial species (Møller *et al,* 1998).

5.2. Transmission of the disease

Transmission of the important enteric infections in pigs is by direct contact with contaminated material (faeces) excreted by affected or carrier animals as, and the route of infection is by ingestion (Pearce, 1999). The chances of susceptible pigs of becoming infected by currently infected pigs are high when a group of animals share the same space, increasing the opportunity for horizontal transmission.

B. pilosicoli may also be isolated from sources such as wild water birds and lake water, however, those isolates did not belong to the same genetic group as assessed by MEE (Oxberry *et al,* 1998). Interestingly, the genetic pattern of those isolates was not similar to that of isolates from pigs, dogs, humans and chickens either. The fact that *B. pilosicoli* was isolated from a water source in a public place and from wild water birds exemplify an epidemiological evidence of potential risk of infection with *B. pilosicoli* for humans and animals. And because *B. pilosicoli* is able to infect a wide range of hosts (section 2 above), it might be feasible that this pathogen colonises other animals that have not been sampled yet.

5.3. Age groups affected

In pigs, *B. pilosicoli* infection is clinically seen between 6 and 12 weeks of age, and the infection is not clinically relevant in suckling pigs (Duhamel *et al,* 1995a). Also, clinical signs of PCS have been observed between 7 and 14 days after moving or mixing weaned pigs (Thomson *et al,* 1998).

5.4. Carriers pigs

It is known that *B. hyodysenteriae* can be transmitted to susceptible pigs by carriers (Alexander and Taylor, 1969), that could maintain *B. hyodysenteriae* viable in their digestive tracts for 70 to 90 days, and the most common source of infection is by in-bought pigs (Windsor and Simmons, 1981). Transmission of infection by carrier pigs was demonstrated in a study where the excretion rate of *B. hyodysenteriae* was monitored in subsequent sampling of pig units. The excretion of *B. hyodysenteriae* by asymptomatic pigs was detected on culture and fluorescent antibody test (FAT) despite pigs receiving prophylactic medication with antimicrobials such as carbadox, dimetridazol or tylosin (Griffin and Hutchings, 1980). Transmission of *B. hyodysenteriae* was suppressed by the routine medication since the recovery of *B. hyodysenteriae* was not high, however *B. hyodysenteriae* was still recovered on culture after subsequent sampling (Griffin and Hutchings, 1980). The results of that study confirmed that asymptomatic pigs are a potential source of infection due to intermittent excretion. Similarly, despite treatment with antimicrobial agents such as tiamulin and lincomycin to control infection with *B. pilosicoli* remained as chronic problem on affected farms (Thomson *et al,* 1998). Thus, carrier pigs may also be an important epidemiological aspect in the infection with *B. pilosicoli*, though it has not been fully studied.

The use of antimicrobial agents routinely in pig farms limits the transmission of SD or reduces the appearance of clinical signs of SD (Griffin and Hutchings 1980; Robertson *et al,* 1992). However, the fact that 37.0 % of farms were serologically positive to swine dysentery despite the use of antimicrobial agents, confirms that using those agents could mask the clinical manifestation of the disease as the managers of those farms had not realised that the disease was subclinically present (Robertson *et al,* 1992).

The presence of carrier animals in SD contributes to persistence of the infection on pig farms. Excretion of intestinal spirochaetes from asymtomatic pigs may be frequent;

therefore, a sequential sampling of herds is justified as a surveillance system for detection of those carriers (Griffin and Hutchings, 1980). In a study carried out in Sweden isolation of *B. hyodysenteriae* from gilts was obtained on culture from three subsequent samplings (3, 8 and 12 months after the original sampling) from a herd known to be affected by SD (Fellström *et al,* 2001). That study showed the importance of subsequent sampling schemes considering the possibility to isolate intestinal spirochaetes from carrier animals.

5.5. Zoonotic potential

Since *B. pilosicoli* infects humans, some studies have investigated the possibility of inter-species transmission by sampling two different susceptible populations. For instance, the prevalence of *B. pilosicoli* infection in humans living in rural communities in different countries has been documented (Barret, 1990; Lee and Hampson, 1992; Trott *et al,* 1997). The reported figure of prevalence in Western Australian Aboriginal communities was 32.6 %, in Oman 22.7 % and in Papua New Guinea from 8.6 to 30.1 % (Lee and Hampson, 1992; Barret, 1990; Trott *et al,* 1997). Because the prevalence in humans from rural communities was found to be higher than the prevalence in domestic animals, it has been suggested that it is more likely that infection might be passed from humans to animals and not the other way round. Also, the infection in humans has been estimated to last approximately four months and has a higher incidence rate (93.4 %). Considering those facts, it has been postulated that transmission of *B. pilosicoli* infecton is more likely to occur from human to human than from human to domestic animals (Trott *et al,* 1997). Moreover, the incidence rate of *B. pilosicoli* infection in humans has been reported as 10.8 % over a period of 6 weeks after a second sampling was carried out from the same human population (Trott *et al,* 1997). Interestingly, the prevalence found in humans less than 2 years old was the lowest in the study by Trott *et al,* (1997). From that study it could be observed that *B. pilosicoli* infection was not as clinically important in that age group as it was for children over two years old or older people.

Considering that the survival of *B. pilosicoli* in pigs faeces was 210 days when kept at <10 °C (Boye *et al,* 2001), therefore, it can deduced that pig faeces represent a potential risk of infection. Also, *B. pilosicoli* seems to be more resistant to environmental conditions than *B. hyodysenteriae*, and since PCS is less dramatic than SD, thus farms infected with PCS would have less chance of being diagnosed due to inapparent clinical signs so, the chances of *B. pilosicoli* being shed and remaining viable in the environment would put humans and pigs at a higher risk of exposure to a potential infection, or reinfection.

5.6. Nutritional factors

The use of antimicrobial agents in the diet of pigs has an effect on the balance of the microbiota of the intestines. It is also believed that the composition of diet influences the intestinal environment, and certain components create the suitable conditions for the colonisation of certain micro-organisms such as intestinal spirochaetes. In the pig production sector cereals are widely used to formulate diets for pigs. Some of those cereals contain considerably high amounts of soluble non-starch polysaccharides (sNSP), insoluble non-starch polysaccharides (iNSP), and resistant starch (RS) fermentable substrate, as components of their cell walls (Englyst and Kingman, 1988). The NSP are molecules of cellulose, hemicellulose, lignin and pectin (Trowell *et al,* 1976). Nutritionists recognise those molecules as fibre which is degraded by bacteria in the large intestine since they are not digested in the small intestine. According to the amount and type of fibre contained in the diet a particular type of microbiota will develop, and consequently a particular type of fermentation, which will influence the microbiological environment. Thus, such conditions will determine temperature, pH, anaerobiosis, osmolality and enzyme activities and total numbers of fermentative bacteria in the large intestine (Siba *et al,* 1996). Therefore it has been postulated that the colonic microbiota may influence the incidence of SD and PCS.

The results from the studies carried out by Durmic *et al* (1998) and Pluske *et al* (1998) strongly suggest that there is a role for the soluble fractions of NSP and for RS in the pathogenesis of *B. hyodysenteriae* infection. This may also apply to infection with *B. pilosicoli* (Trott *et al,* 1996a), since they can both be found causing mixed infection in the large intestine of pigs (Thomson *et al,* 1998).

The changes of the bacterial populations in the large intestine of pigs after fed with different diets and variable contents of total NSP, sNSP and RS have been assessed by molecular methods. Characteristic terminal RFLP (T-RFLP) patterns are generated according to the bacterial communities composition (Clement *et al,* 1998). For instance, a study showed that the T-RFLP patterns from the microbiota of pigs fed on the same diet were identical. Whereas the T-RFLP patterns of the intestinal microbiota of pigs fed on different diets were distinct, indicating that the bacterial community was influenced by the diet composition (Leser *et al,* 2000). Interestingly, after infection with *B. hyodysenteriae*, the T-RFLP patterns of the intestinal bacterial communnity in the challenged pigs was distorted as result of the pathological condition produced by *B. hyodysenteriae* (Leser *et al,* 2000).

6. Diagnosis

The development of an accurate and efficient laboratory diagnostic test for the identification of the causative agents of SD or PCS has been a significant target to achieve. Because of the biology of such diseases, the diagnosis sometimes becomes so complicated that more than one test must be carried out before the diagnosis is definitive.

The diagnosis of intestinal spirochaetal infections in pigs should be based on the clinical history of the affected herd, clinical signs of the disease, identification of the micro-organism on smears from rectal swabs or faecal samples, postmortem examination,

and growth of the micro-organism on bacteriological plates in the laboratory. None of the above tests should be used alone as conclusive, since the results from different tests are complementary to each other. With the discovery of various species of intestinal spirochaetes affecting the intestinal tract of pigs, the diagnosis has become more complicated. Most of the diagnostic tests for intestinal spirochaetes have been developed for *B. hyodysenteriae*, and then adapted to other species of intestinal spirochaetes.

6.1. Traditional methods for diagnosis of *Brachyspira pilosicoli*

Traditional methods for diagnosis of intestinal spirochaetal infections in pigs are based on isolation of the micro-organism coupled with clinical signs, lesions and observation of spirochaetes on stained smears. Differential diagnosis of SD at culture level seems to be relatively easy since it is well established that *B. hyodysenteriae* produces a characteristic strong β-haemolysis. However, the isolation of *B. hyodysenteriae* is more difficult when pigs become carriers of the disease. Moreover, the diagnosis of an intestinal spirochaetal infections in pigs by traditional methods becomes more complicated since other *Brachyspira* species may be involved in a mixed infection with other *Brachyspira* species given rise to unusual biochemical reactions (Olson and Fales, 1983; Hommez *et al,* 1998).

Several culture media have been developed for the isolation of *B. hyodysenteriae* and have been adapted for the isolation of other intestinal spirochaetes. As a fastidious bacteria, intestinal spirochaetes have been most effectively isolated by the use of selective culture media to inhibit other intestinal bacteria. Generally, culture media for intestinal spirochaetes are based on trypticase soy agar supplemented with blood and various combinations of antibiotics (Table 1.4). Incubation temperatures at the range of 37 to 42 °C have been used for intestinal spirochaetes (Jensen, 1997).

Another method reported an improvement in the sensitivity of the isolation of *B. hyodysenteriae* by sliced agar (Olson, 1996). The method consisted in making 10 cuts of 1 cm of separation each on agar plates of two slightly different selective media (TSA-S400 and a medium containing spectinomycin, colistin, vancomycin, spiramycin and rifampicin). The sliced agar plates produced better results in terms of the visibility of the haemolysis at the edges of the cut. The recovery of *B. hyodysenteriae* on sliced agar from pigs with chronic diarrhoea was higher than using non-sliced plates. Also, *B. hyodysenteriae* sometimes grew without any contaminants when cultured on sliced agar plates which saved labour and time for diagnosis (Olson, 1996).

Recently, another selective medium for the isolation of *B. hyodysenteriae* was adapted from a medium developed for the isolation of *B. pilosicoli* (Calderaro *et al,* 1997). One of the main points of that method consists of the suspension of samples either as faecal material or colon scrapings into brain heart infusion (BHI) broth suplemented with 10 % foetal calf serum, 400 μg/ml spectinomycin and 15 μg/ml rifampin. By treating samples that way for 15 to 30 minutes allows the antibiotics to act against the pig normal flora. This selective medium is based on agar base No. 2 supplemented with 7 % defibrinated horse blood, beef extract 3 g/l, bacto peptone 5 g/l, and containing 400 μg/ml spectinomycin and 30 μg/ml rifampin (Calderaro *et al,* 2001).
Regarding liquid media, the growth of porcine intestinal spirochaetes has been done successfully in trypticase soy broth supplemented with 10.0 % foetal calf serum which increased between 10 to 1000 colony forming units (CFU) per ml of broth (Kinyon and Harris, 1974). In another study, six different liquid media were used for the propagation of *B. hyodysenteriae*, pleuro-pneumonia-like organism broth (PPLO), trypticase soy broth (TS), veal infusion broth (VS), brain-heart infusion (BHI), volatile fatty acids yeast extract trypticase peptone (VTY) and heart infusion broth (HS). All the broths were supplemented with 10.0 % inactivated foetal calf serum and 0.2 % glucose. The broths BHI and VS produced the highest cell numbers for the three different reference strains at the shortest times of incubation 22 to 24 hrs and 20 to 32 hrs (Stanton and Lebo, 1988).

Table 1.4. Selective culture media for the isolation of intestinal spirochaetes.

Medium	Based component	Supplement	Antibiotics added	Reference
TSA-S400	TSA	10.0 % sheep blood	400 μg/ml spectinomycin	Songer et al (1976)
TSA-CVS	TSA	10.0 % sheep blood	25 μg/ml colistin 25 μg/ml vancomycin 400 μg/ml spiramicin	Jenkinson and Wingar (1981)
BJ	TSA	5.0 % sterile citrate bovine blood 5.0 % pig faecal extract	200 μg/ml spectinomycin 6.25 μg/ml colistin 6.25 μg/ml vancomycin 25 μg/ml spiramycin 12.5 μg/ml rifampycin	Kunkle and Kinyon (1988)

Diagnostic methods for intestinal spirochaetes in pigs based on the isolation of the micro-organisms in bacteriological culture could be tedious since these micro-organisms are fastidious and differentiation of species may take several steps. Hommez *et al,* (1998) argue that reliance on a single test to diagnose digestive disorders in pigs caused by *Brachyspira* species may lead to erroneous identification of the agents involved. After samples are positive on culture, the procedure for the identification of the species should include the type of haemolysis to differentiate *B. hyodysenteriae* from other species, and biochemical tests such as indole and hippurate hydrolysis are essential for the identification of WBHIS. Indole negative and hippurate positive are characteristic reactions produced by *B. pilosicoli,* and indole positive reactions identify *`Brachyspira-Serpulina' intermedia,* whereas indole and hippurate hydrolysis negative reaction identify both of the non-pathogenic species *B. innocens* and *`Brachyspira-Serpulina' murdochii* (Fellström *et al*, 1997; Hommez *et al,* 1998). A few more biochemical reactions that contribute to the accuracy of the identification of particular species, include α and β-glucosidase and α-galactosidase. *B. pilosicoli* produces negative reactions for α- and β-glucosidase, and variable reactions for α-galactosidase. Variation

in biochemical reactions from *Brachyspira* species can be expected as a result of mixed infections and further investigation may be needed (Hommez *et al,* 1998). Despite complications, the diagnosis of porcine intestinal spirochaetes could be done by simple bacteriological culture coupled with few biochemical tests (Hommez *et al,* 1998).

6.2. Serological tests

A variety of serological tests have been developed for the diagnosis of intestinal spirochaetes, particularly for *B. hyodysenteriae.* During the 1970s and 1980s diagnostic tools for intestinal spirochaetal infections in pigs were basically the direct and indirect flourescent antibody tests (FAT and IFAT, respectively). However, there was some controversy about the results produced by these and other tests based on detection of antibodies or antigens due to either poor specificity or sensitivity; therefore, results were not always regarded as conclusive. Later, immunodiagnosis of *B. hyodysenteriae* was based on agarose gel immunodiffusion using extracted LPS and antisera raised in rabbits. Since extraction of LPS requires high numbers of bacteria, microagglutination (MAT) test was then proposed for the immunodiagnosis of *B. hyodysenteriae* using whole cell preparations (Burrows and Lemcke, 1981; Diarra *et al,* 1994). Thus, MAT was reported to be the definitive test for identification of *B. hyodysenteriae* in the UK (Burrows and Lemcke, 1981). However, this is no longer used due to lack of specificity. A more recent study on serology of intestinal spirochaetes by MAT found cross-reactivity when using whole-cell formalinized preparations (Diarra *et al,* 1994). Cross-reactivity was significantly reduced by boiling the whole cell preparations, suggesting that the antigens that participate in cross-reactivity might be heat labile (Diarra *et al,* 1994).

A comparative study between ELISA and MAT using LPS (as antigen) for detection of antibodies against *B. hyodysenteriae* showed that ELISA detected antibodies between 7 and 10 days post-infection, whereas MAT detected antibodies after two weeks post-

infection. High antibody titres were still detected at 19 weeks post-infection by ELISA, whereas detection of antibody titres by MAT tended to fall from three weeks post-infection onwards. Those serological tests were useful as diagnostic tools, however cross-reaction still occurred between serotypes (1 and 2) of *B. hyodysenteriae* (Joens *et al*, 1982).

In another study, the detection of antibodies from 19 herds (1640 sera) affected by SD and 11 herds (1519 sera) free from SD was compared using two different serologic tests, MAT and ELISA coupled with bacteriological isolation. Of the 11 herds free from SD, no pigs were detected positive in any herd by MAT, whereas ELISA detected positive pigs in 2 of those 11 herds. Of the 19 herds affected with SD, MAT detected 12 positive herds, while ELISA picked up 18 positive herds. Consequently, the detectable infection rates were higher for ELISA (18.0 %) using cell preparations of two reference strain antigens than MAT (3.0 %) using one reference strain as antigen. Although, in that study ELISA was shown to be superior in terms of sensitivity, it was still not suitable for individual animal testing but recommended for herd screen testing (Egan *et al,* 1983).

Another study evaluated the sensitivity of serologic tests such as indirect haemagglutination (IHA), passive haemolysis (PH), coagglutination and MAT for detecting antibodies to *B. hyodysenteriae* and *B. innocens* from natural and experimental infection (Diarra *et al,* 1995). Of those serological tests, MAT was the only one that detected antibodies in infected pigs, and none of the non-infected pigs was detected positive. Although, higher antibody titters were detected by MAT using non-boiled antigen, cross-reaction remained occurring between *B. hyodysenteriae* and *B. innocens* and between the different *B. hyodysenteriae* serotypes which was reduced by boiling the Ag cell preparations. Moreover, with the Ag prepared that way MAT was able to differentiate the sera of pigs infected with either *B. hyodysenteriae* or *B. innocens*, and infected herds were detected positive by MAT at the level of 70.0 to 95.0 %. Also, MAT detected positive 50.0 % of the herds with previous history of SD and 10.0 % of the herds apparently free of SD (Diarra *et al,* 1995).

The colonisation of the intestinal tract of pigs with WBHIS including the non-pathogenic intestinal spirochaete *B. innnocens* influences the results obtained by FAT, and renders the test non-specific. Therefore, veterinary diagnosticians developed a FAT for the diagnosis of *B. hyodysenteriae* by using an absorbed serum with *B. innocens* to increase specificity (Hunter and Saunders, 1977), but in recent years better diagnostic tools have been developed (see below).

The isolation and proper identification of intestinal spirochaetes, particularly, those causing SD (*B. hyodysenteriae*) and PCS (*B. pilosicoli*) will provide the elements to understand more clearly the epidemiology of such diseases.

6.3. Molecular methods for the diagnosis of infection by intestinal spirochaetes in pigs

The need for efficient identification and differentiation of intestinal spirochaetes has pushed diagnostic procedures towards the use of molecular methods. Bearing in mind that the isolation of these micro-organisms is laborious and cross-reactivity between species occurred in serologic tests. Molecular methods such as PCR have been reported as another tool available for the identification of intestinal spirochaetes which could be more sensitive, specific and rapid. Those detection methods have required identification of specific genomic targets.

One of the first reports on PCR for identification of *B. hyodysenteriae* from cultured cells and clinical material such as pig faeces, rectal swabs or mucosal scrapings was described by Elder *et al,* (1994). That PCR generated an amplicon only from DNA of *B. hyodysenteriae* serotypes 1 to 9, showing high levels of specificity since the DNA from other *Brachyspira* spp., *Spirochaetales* or enterobacteria such as *Salmonella* spp., *Campylobacter* spp., *Bacteroides vulgatus* and *E. coli* was not amplified (Elder *et al,*

1994). In that study, PCR was complementary to hybridisation with a DNA probe specific for *B. hyodysenteriae* showing high specificity as that probe hybridised only with DNA of *B. hyodysenteriae*. The sensitivity of detection of that PCR was at the level of 1 to 10 cells/0.1 g of faeces or 1000-fold more sensitive than culture (which seemed to be a big advantage for detecting low numbers of bacteria). Additionally, that PCR could be completed in one day which is another advantage over bacteriological culture which usually takes between 2 to 6 days (Elder *et al,* 1994).

The identification of *B. pilosicoli* and *B. hyodysenteriae* by specific probes used in *in situ* assays has been developed in order to shorten the time for diagnosis of these two pathogens. Hybridisation *in situ* of formalin-fixed tissue samples showed that the probes Hyo1210 specific for position 1210-1228 of the 23S rRNA gene, and Pilo209 specific for position 209-226 of the 16S rRNA gene and Pilo1405 specific for position 1405-1424 of the 23S rRNA gene (Boye *et al,* 1998), were able to identify specifically *B. hyodysenteriae* and *B. pilosicoli* on histological preparations from diseased animals and on preparations of cultured cells of the reference strains and also from field isolates of the two species. Since no hybridisation signal was found with any of the controls, and *B. pilosicoli* and *B. hyodysenteriae* were properly identified in tissue sections containing as few as 10 target cells among the normal microflora of the pig intestines, that method was regarded as highly sensitive for the diagnosis of *B. hyodysenteriae* and *B. pilosicoli* as no hybridisation reaction was observed with any of the controls (Boye *et al,* 1998). An advantage of the *in situ* hybridisation assay targeting rRNA instead of DNA is that copies of the rRNA are produced during the exponential growing of bacterial cells; therefore, a higher sensitivity may be achieved by targeting rRNA.

Another PCR technique was found to be 100 % sensitive when applied to a collection of isolates of *B. pilosicoli* isolated from pigs and humans from different geographical locations. The PCR was based on two primers targeting sequences of the 16S rRNA gene of *B. pilosicoli* which generated a product of 1300 bp approximately from all the

B. pilosicoli isolates tested. No PCR product was generated from any other *Brachyspira* species than *B. pilosicoli*, showing high specificity, and the sensitivity was reported at the level of 250 cells per reaction (Park *et al,* 1995). However, the DNA of *Serpulina jonesii* was amplified by that PCR test, adding weight to the hypothesis that the species *Serpulina jonesii* was closely related to *B. pilosicoli* (Park *et al,* 1995). A disadvantage of that PCR test was that primary bacterial cultures were still needed to perform this PCR assay, increasing with this the time for diagnosis and labour (Park *et al,* 1995).

Another PCR was designed for the specific identification of *B. pilosicoli* based on the use of two primers flanking a region between the variable regions V2, V3 and V4 of the 16S rRNA gene of *B. pilosicoli*. The primers target the sequences at positions 151 to 179 and 1060 to 1083 on the 16Sr RNA gene of *B. pilosicoli*, and were selected in a way to differentiate *B. pilosicoli* (biochemical group IV) from other species of intestinal spirochaetes by generating a PCR fragment of 930 bp size (Fellström *et al,* 1997). The specificity of that PCR was confirmed by no amplification of the DNA of any other of the species of intestinal spirochaetes biochemical groups I to III, neither of *Salmonella* spp, *Campylobacter* spp. or *E. coli*. Interestingly, the PCR results of the reference strains P43/6/78 and M1, and field isolates from Belgium, Denmark, Scotland and Sweden were in agreement with their biochemical classification (Fellström *et al,* 1997).

A PCR targeting sequences of the 23S rRNA gene of *B. hyodysenteriae, 'Serpulina-Brachyspira' intermedia* and *B. pilosicoli* has also been used for specific identification of these species (Leser *et al,* 1997). That PCR assay generated DNA fragments of 1321 bp, 143 bp, and 1027 bp specific for *B. hyodysenteriae, B. pilosicoli* and *'Serpulina-Brachyspira' intermedia*, respectively. Those PCR fragments were species specific since no DNA amplification was generated from other '*Serpulina-Brachyspira'* spp nor other enteric bacteria such as *Salmonella typhimurium, E.coli, Clostridium perfringens* or *Campylobacter coli.* The sensitivity of detection of *Brachyspira* cells in spiked faeces was at the level of 5×10^5 cells/g of faeces, and a significant advantage was that faecal samples could be processed in 1 day with high specificity (Leser *et al,* 1997).

Atyeo *et al* (1998) developed another PCR the identification of *B. hyodysenteriae* by targeting a specific sequence of 1700 bp to generate a PCR product of 180 bp, and for the identification of *B. pilosicoli* using the primer Ac1 to target a sequence on the 16S rRNA gene to generate a product of 1300 bp described by Park *et al* (1995). The sensitivity of that PCR was evaluated using spiked pig faeces, clinical samples and faecal samples from experimentally challenged animals. That PCR identified specifically *B. hyodysenteriae* by the characteristic fragments of 180, 600 and 750 bp, and *B. pilosicoli* by the characteristic fragment of 1300 bp (Ayteo *et al,* 1998). The specificity of that PCR was confirmed since no DNA amplification products were generated from any other bacteria. The levels of detection of *B. hyodysenteriae* and *B. pilosicoli* in spiked faeces were reported at the level of 10^3 to 10^4 cells per 0.2 g of faeces, and the sensitivity of detection from clinical samples of pigs naturally infected using that PCR was higher 83.0 % (15/18) than direct culture 50.0 % (9/18) (Ayteo *et al,* 1998).

Another PCR for the identification of five species of intestinal spirochaetes in faecal samples of pigs was developed and compared to bacteriological culture. *B. hyodysenteriae, B. pilosicoli, B. innocens, 'Brachyspira-Serpulina' intermedia* and *'Brachyspira-Serpulina' murdochii* were specifically identified by that PCR which generated a characteristic DNA fragment from each *Brachyspira* species, and also a DNA fragment characteristic of the genus *Brachyspira* (Moalic *et al,* 2001). The sensitivity of detection of that PCR assay was at the level of 200 cells per reaction, and high specificity was confirmed by absence of DNA amplification from 28 species of pathogenic and non-pathogenic bacteria that colonise the intestines of pigs. Interestingly, that PCR amplified the DNA of *B. pilosicoli, B. innocens* and *'Brachyspira-Serpulina' intermedia* from a single sample as part of a mixed colonisation. Although, the difference in terms of sensitivity as compared with culture was not significant, the advantage of using that PCR assay in diagnosis was the time of

sample processing which could be done in 1 day, and therefore giving time to treat the affected animals (Moalic *et al,* 2001).
PCR has been proved to be a useful method for routine diagnosis of SD and PCS, however more sensitive methods are needed for detection of carrier pigs.

7. Control

The control of SD and PCS has been basically done by in-feed medication as a prophylactic practice or treatment with antibiotics at therapeutic doses in clinical outbreaks. Other measures for controlling pig infectious diseases include husbandry methods such as all-in-all-out, isolated weaning, multiple site production and medicated early weaning, coupled with cleaning and disinfection practices (Waddilove and Blackwell, 1997). The use of vaccines has not had a great success for controlling SD and little information is available in this regard for the control of other intestinal spirochaetal infections like PCS. It is believed that serum of pigs recovered from SD is protective due to an immune response developed to LPS of *B. hyodysenteriae*; therefore, pigs recovered from SD become resistant to further experimental infection (Olson, 1974). It has also been suggested that disease could be recurrent due to a non fully protective immune response since pigs recovered from SD developed the disease again between 4 to 10 days after the first episode (Kinyon *et al,* 1977). However, it has been suggested that an immune response against *B. hyodysenteriae* would be serotype specific since intestinal loops challenged with a range of serotypes still developed infection (Joens *et al,* 1982).

A study by Alexander *et al* (1980) evaluated the medicated early weaning (MEW) system. A total of 105 pregnant sows were divided into groups according to antibiotic treatment for its effectiveness on the vertical transmission of SD or PCS and other pig infectious diseases. In the herd of origin that provided the pregnant sows to the experimental herd evidence of the presence of intestinal spirochaetes was confirmed on

culture but no clinical signs had been observed, perhaps due to the use of antibiotics. The medication programme for sows and piglets included tiamulin, and combinations of trimethoprim and sulfadoxine or trimethoprim and sulphadiazine. Sows were injected at high doses before farrowing and then orally medicated in feed and water until the early weaning took place (5 days after farrowing), and piglets were also injected soon after birth and orally medicated. Samples from piglets to monitoring pathogens were taken at 5 days, 8-11 weeks, and at slaughter. Intestinal spirochaetes were isolated from faecal samples and colonic scrapings of pigs at slaughter at a higher rate 85.0 % (17/20) from untreated pigs than that of pigs treated with antibiotics 17.6 % (3/17). *Brachyspira* cells were also recovered from 100 % (6/6) of pigs in the herd of origin ageing 8-16 weeks. Interestingly, *Brachyspira* cells were not isolates from young pigs (up to 11 weeks) of the MEW trial, suggesting that the antibiotics treatment had an effect on the delay of excretion of intestinal spirochaetes. Although, MEW groups did not show clinical signs of intestinal spirochaetal infection through the experiment, no conclusions were made on whether the MEW was effective in breaking the cycle of infection of intestinal spirochahetes, or the isolates from pigs of the MEW group may have arisen from a new infective source, perhaps transmitted into pigs by rats (Alexander *et al,* 1980).

By 1977 in the German Democratic Republic an eradication programme for SD was in progress based on the systemic administration of metronidazol or tylosin for a period of 12 days (7 consecutive days followed by another 5 days with a 14 day gap) (Blaha *et al,* 1987). The affected herds applied strict cleaning and disinfection, and restricted the movement of pigs. Although, the antibiotic treatment was effective in controlling outbreaks of SD, subclinical cases were still identified and the incidence rate did not decrease. A surveillance system to monitor herds with latent infection was therefore established. The sampling scheme consisted of taking 100 faecal samples of pigs of all age groups from breeding herds and testing them by phase-contrast microscopy. The disease free status of the herd was obtained after three consecutive negative samplings (100 animals each sampling) at monthly intervals. The eradication programme for SD based on treatment with the antibiotics metronidazol or tylosin was effective after two

years since the 38 herds detected with latent infections were free of the disease by then, and the whole region was declared free of SD. However, due to concern over safety with the use of metronidazol and an increase in resistance to tylosin, that eradication programme was reviewed, and the use of different antibiotics such as tiamulin was proposed (Blaha *et al,* 1987). The eradication of SD from a 590 sow herd affected with SD was succeful using tiamulin at 10 μg/kg of bodyweight as treatment for 5 consecutive days to all the pigs (except piglets under three weeks of age). The tiamulin therapeutic scheme described was effective since clinical signs of SD disappeared after two days of treatment. The success of eradication of SD from that herd was a combination of tiamulin treatment together with strict cleaning and disinfection, and a rodent extermination programme. No spirochaetes were seen in faecal samples analysed by phase-contrast microscopy and indirect immunoflourescence on subsequent occasions for a period of two years (Blaha, *et al,* 1987).

Several studies have been done to test the sensitivity of *B. hyodysenteriae* to different antimicrobials under laboratory conditions. For instance, (Molnár, 1996) reported the sensitivity of 322 isolates of *B. hyodysenteriae* from Hungary over three periods from 1978 to 1987, 1983 to 1987, and 1988 to 1992 against several antimocrobial agents including carbadox, dimetridazol, tylosin, lincomycin, tiamulin, monensin, and sedecamycin. The results of that study showed that as a result of tylosin being used as the antibiotic of choice for the treatment SD and being included in diets of pigs as an additive since the 1980s, an increase of resistance to tylosin over time was observed. The percentages of resistance to tylosin for the three periods over time were 11.0 % (10/91), 40.5 % (49/124), and 67.0 % (78/117), respectively, with minimal inhibitory concentration (MIC) values 0.10-100 μg/ml. The rate of moderate sensitivity also increased and the rate of sensitivity decreased considerably from 71.4 % (1978-1982) to only 8.6 % (1988-1992) (Molnár, 1996). Resistance rate to lincomycin also increased chronologically from 11.0 % to 22.5 % and to 27.5 %, respectively, with MIC values 0.20-100 μg/ml, lincomycin was the second antibiotic of election for the treatment of *B. hyodysenteriae* in Hungary. Dimetridazol has also been used widely for the treatment of SD, the most remarkable change in the study of Molnár (1996) was on the rate of

moderate sensitivity which increased chronologically from 13.2 % (12/91) to 32.0 % (40/124) and to 44.2 % (52/117), respectively, and the sensitive rate was at the level of 46.0 %, with MIC values 0.10-50 μg/ml. Late in the 1980s and during the 1990s tiamulin has been widely used for the treatment of SD as the antibiotic of choice, the results of sensitivity to this antibiotic were 100 % for the period 1983-1987, and 82.9 % (97/117) for the period 1988-1992. This showed a slight increase in resistance from 0.0 % (0/16) to 7.7 % (9/117), respectively, with MIC values of 0.05-50 μg/ml. Although, carbadox has also been used widely in pig feed for number of years, it still showed high efficacy to *B. hyodysenteriae* as the sensitivity rate was 100 % for the three periods examined with MIC values of 0.5-0.40 μg/ml (Molnár, 1996). Isolates of *B. hyodysenteriae* have also been tested for sensitivity to tiamulin in the UK. The MIC range was lower than that found by Molnár (1996), being 0.015-0.25 μg/ml, and the MIC_{50} and MIC_{90} were reported to be 0.062 and 0.25 μg/ml with no resistance to tiamulin being evident at that stage (Dalziel, 1996). However, this has not been assessed recently.

In another study the MIC and the minimum bactericidal concentration (MBC) for *B. hyodysenteriae* for 23 isolates from Australiaand 7 isolates from other countries were obtained by macrobroth dilution (Buller and Hampson, 1994). Most of the isolates in that study showed resistance to tylosin and lincomycin, the MBC and MIC rates for tylosin were 16->128 μg/ml and 16->128 μg/ml, respectively, and for lincomycin 1.0->128 μg/ml for both, respectively. The MIC_{50} and MIC_{90} values for tylosin were 128 and >128 μg/ml, respectively, and for lincomycin 64 and 128 μg/ml, respectively. Comparatively the MBC and MIC rates for tiamulin and dimetridazole had the same values for both, 0.25-128 μg/ml. The MIC_{50} and MIC_{90} values for tiamulin were 1.0 and 8.0 μg/ml, respectively, and for dimetridazole 1.0 and 4.0 μg/ml, respectively. Most of the isolates were susceptible to lower concentrations of tiamulin and dimetridazole but one isolate from Australia had a remarkable high MIC of 128 μg/ml. Isolates from different parts of the world showed a wide range of susceptibility or resistance to the

antimicrobial agents tested in that experiment, indicating that resistance might have been developed by *B. hyodysenteriae* gradually (Buller and Hampson, 1994).
The testing of antibiotics for the treatment of intestinal spirochaetal infections *in vivo* has also been documented (Raynaud *et al,* 1981). An experiment in which single antibiotics carbadox (50 μg/kg of feed), niathiamide (150 μg/kg of feed) and olaquindox (100 μg/kg of feed), or antibiotic combinations of carbadox (50 μg/kg of feed) + sulfamethazine (100 μg/kg of feed), and chlortetracycline (100 μg/kg of feed) + sulfamethazine (100 μg/kg of feed) + Penicillin (50 μg/kg of feed) were included in pig feed to evaluate their efficacy to infection with *B. hyodysenteriae* in a challenge experiment compared to control non-medicated pigs. From that experiment, the morbidity and mortality of the non-medicated pigs was as high as 100 and 87.5 %, respectively. Only the pigs fed on diets with carbadox or the combination carbadox + sulfamethazine were protected against challenge infection, whereas the pigs fed on the diets with olaquindox were partially protected since after removal of the medication the morbidity was high and even mortality was observed. Pigs fed on diet with that combination of antimicrobial agents (carbadox + sulfamethazine) had better performance [0.413 g average daily gain (ADG) and 3.47 feed conversion (FC)] than pigs fed on diet with carbadox alone (0.364 g ADG and 3.57 FC) (Raynaud *et al,* 1981).

Antibiotics may therefore be used successfully to prevent and treat intestinal spirochaetal infections, however alternative methods for control such as vaccination is also under development.

Commercial vaccines have been developed and tested for the control of SD. For instance, in an expermient an inactivated whole cell vaccine was injected intramuscularly into pigs two or three occasions (Hampson *et al,* 1993). Unvaccinated control pigs were also challenged. Pigs developed an immune response to the vaccine in which antibody titres increased after the second and third vaccination as measured by ELISA using plates coated with purified LPS as antigen. Pigs in the group given two vaccinations and challenged with an infective dose containing 10^8 cells of

B. hyodysenteriae resulted in only 1/6 of the vaccinated pigs develop haemorrhagic diarrhoea 9 days after challenge and started shedding spirochaetes two days after challenge, whereas 5/6 of the unvaccinated pigs developed the clinical signs of SD (Hampson *et al,* 1993). Pigs given three vaccinations resulted in 2/8 of the vaccinated pigs developing SD, while 7/8 of the control unvaccinated were affected by SD. Surprisingly, two of the vaccinated pigs and the remaining unvaccinated pigs from one of the experimental groups developed diarrhoea four weeks after challenge. An antibody response was observed in vaccinated pigs which provided partial protection as compared to unvaccinated control group, although the vaccinated pigs were still susceptible to infection with *B. hyodysenteriae* when challenged (Hampson *et al,* 1993).

Another study reported the use of an experimental and a commercial vaccine inoculated by intramuscular injection into pigs at 8 weeks of age, the pigs were challenged with strains of different serotypes 4 and 5 weeks later, using as infective material the colon of a pig affected with SD. Although pigs inoculated with both, the commercial and experimental vaccines developed an immune response as high antibody titre levels were detected pigs developed the characteristic clinical signs of SD. Moreover, the clinical signs on vaccinated pigs developed earlier than in pigs of the control group. The mortality rates of the vaccinated and unvaccinated groups were at the level of 32.0 % (8/25) and 40.0 % (6/15), respectively, but the lesions at postmortem examinations were more severe in the vaccinated group than those of the unvaccinated group. Diarrhoea was still observed in the surviving pigs after 5 weeks of challenge and *B. hyodysenteriae* was isolated from a sentinel pig that was put into contact with those pigs. The authors proposed that higher severity in vaccinated pigs might be through hypersensitivity reactions to undefined antigens of *B. hyodysenteriae*, and despite high levels of antibodies disease still occurred (Olson *et al,* 1994).

Although comprehension of intestinal spirochaetes has improved over the past few years, detection (particularly of carrier pigs), differentiation and control of these infections present some difficulties.

The aims of this study are firstly, to determine the risk factors that contribute to the presence of PCS in pig farms within the UK. The epidemiological approach of this study is based on the calculation of odds ratios using χ^2 test for a case-control study using data collected from pig farms. Secondly, to assess the immunomagnetic separation (IMS) for the detection of target cells (*B. pilosicoli* and *B. hyodysenteriae*) in pig faecal samples with the objective in mind of applying this detection method to clinical samples for the detection of low numbers of infective bacteria shed by carrier pigs. As porcine intestinal spirochaetes are fastidiuos and a large number of bacterial species are contained in faecal samples a selective culture medium is used to favour the isolation of those target cells. Thirdly, a molecular method based on PCR using arbitrary primers for genetic comparison of the whole genome of field isolates of *B. pilosicoli* is used. Various factors may affect the reproducibility of the AP-PCR assay; therefore, optimisation for each of the primers used is needed on the lines of reagents and cycling temperatures. Finally, a method for controlling PCS is evaluated by testing the efficacy of seven disinfectant-sanitisers to inhibit the growth of *B. pilosicoli* in the presence of organic material as pig faeces.

Chapter two

EPIDEMIOLOGICAL INVESTIGATIONS INTO RISK FACTORS FOR PORCINE COLONIC SPIROCHAETOSIS

Introduction

Epidemiological surveys have shown that enteric diseases in pig farms have a significant effect on the health of pigs, and certain husbandry practices have been found to contribute to the incidence of enteric diseases, especially infectious colitis. Epidemiological studies for assessing risk factors associated with the presence of swine dysentery (based on odds ratios-OR) found that one of the most relevant factors was swine dysentery previously diagnosed in a herd (Robertson *et al*, 1992). In that study, farms that had been diagnosed with the disease were 15.5 more times likely to have another outbreak of SD. Other factors such as the breeding stock bought from sale yards, grower pigs purchased from various sources, visitors allowed into the farm, and rodent presence in the farm were 5.1, 3.9 and 3.3 more times at risk of developing swine dysentery, respectively. That survey did not find herd size (more than 500 sows) as a factor associated with the occurrence of swine dysentery. On the other hand, the factors that were found to be protective against the occurrence of SD included all-in-all-out, automatic feeding, cleaning and disinfection, and the use of antimicrobials in drinking water or in the feed (Robertson *et al,* 1992).

Asymptomatic pigs are important for transmission of SD so, carrier pigs present a high risk of introducing infection to susceptible pigs (Griffin and Hutchinson, 1980).

Epidemiological studies for the identification of factors associated with the occurrence of scour problems in grower-finishing pigs in the UK have been done by questionnaire methodology (Pearce, 1999). From that study some of the factors identified as associated with scour problems were farms with partially slatted floors and farms with wet feeding systems, while, cleaning and disinfection was identified as protective factor (Pearce, 1999). However, only 40.0 % (42/105) of the farms from that study applied disinfection practices.

Association between pig pathogens has also been suggested, for instance, co-infection between *Fusobacterium necrophorum* and *B. hyodysenteriae* in the course of SD has been identified (Alexander *et al,* 1976). That synergism has also been supported by studies on diet composition as *Fusobacterium necrophorum* was always observed in pigs fed on diets with untreated wheat (Durmic *et al,* 2000).

Another epidemiological study for the causes of porcine colitis found that *B. pilosicoli* could occur as a co-infection together with other pathogens such as *Yersinia pseudotuberculosis, Salmonella spp., L. intracellularis* and *B. hyodysenteriae* (Thomson *et al,* 1998). In that study mixed infections were reported to have greater impact on pig growth rates than infections with *B. pilosicoli* alone (Thomson *et al,* 1998).

Another factor of feed which may be important is digestibility. It has been documented that pigs fed with diets that contain highly digestible ingredients that are digested in the small intestine are less likely to develop SD (Siba *et al,* 1996). The principle is that highly digestible diets have less non-starch polysaccharides (NSP) and resistant starch (RS), and these types of diets will reduce the amount of the substrate for bacterial fermentation in the large intestine.

The aim of the present study was to determine risk factors for PCS by performing epidemiological investigations in a case-control study of two different data sets collected by veterinary surgeons from visits to pig farms.

Material and Methods

Study design

Study 1 included data collected during 1996 to 1998 by one veterinary surgeon. The data collected during farm visits to ensure the health status or disease investigation included seven variables regarding type of herd, herd size, source of pigs, type of feed, flooring type, production system and cleaning and disinfection between batches. Units with and without colitis problems associated with *B. pilosicoli* were matched on the basis of herd size or type of enterprise (farrow to finish or finisher only) to set a case-control study.

Study 2 was designed for personal farm visits to collect information by completion of a questionnaire which included 81 questions regarding the disease history of the unit, origin of replacement stock, feeding and watering, nutrition, cleaning and disinfection practices and general management. Farms included in this study were part of a project on causes and epidemiology of porcine colitis carried out by the Scottish Agricultural College during 1999-2000. The visits were carried out by two veterinary surgeons. The questionnaire was pre-tested before the start of the study to ensure a consistent approach to data collection. Data from farms with or without infectious colitis were subjected to detailed analysis (case-control study) to determine the relevant risk factors associated with the disease.

Study population

For study 1 and study 2, the selected herds were farrowing to finish located in different regions of Scotland. The health status of the selected farms was based on a previous sampling. Farms diagnosed with infectious colitis by isolation of intestinal spirochaetes on culture and biochemical identification of the type of intestinal spirochaete from samples of post-weaning and growing pigs were regarded as 'infectious colitis' farms, and isolation negative farms were regarded as 'control' farms. The farms were assigned

a letter coding identification. Further clinical and laboratory investigations were carried out to determine infectious causes of colitis in farms included in study 2.

Sample size and procedures

Study 1. A total of 44 farms with infectious colitis and 44 control farms were included in this study.

Study 2. A total of 47 farms were included in this study. Postmortem (PM) examinations were carried out on 3 pigs (weighing between 15 and 45 kg with clinical diarrhoea) per farm, and faecal samples of 9 pigs (between 6 and 9 weeks of age) per farm were taken to determine the causal agent of colitis for the infectious colitis farms (IC farms), or to corroborate the disease-free status of control farms. Faecal samples were subjected to bacteriological investigations for *Salmonella* spp., *Yersinia* spp. and *Brachyspira* spp., and PCR testing for *Lawsonia intracellularis*. Also parasitological investigations by flotation method and worm egg count by microscopy in McMaster counting chamber, and coccidia examinations were carried out. Representative samples of small and large intestine were also taken for histopathological examination.
On the basis of clinical and laboratory findings the farms were classified as follows: 20 case farms with infectious colitis (IC), 17 farms with non-specific colitis (NSC), which had pathological colitis but not infectious one, and 10 control farms with no clinical or pathological evidence of colitis or any of the above enteropathogens detected on culture. Care was taken to ensure that NSC and control farms had not been using antibiotics to control infectious diseases and that the batches of pigs tested had not received medication.

Data collected

For study 1, the information of farms included:

-type of herd, a= weaner producer, b= breeder/finisher, c= finisher

-herd size, a= >300 sows, b= <300 sows, c= >500 finishers, d= <500 finishers (c and d apply to finisher farms only)
-source of breeding stock, a= single source, b= various sources, c= home bred replacements
-feed type, a= pelleted, b= meal, c= home mix (all meal)
-housing (flooring), a= fully slatted, b= solid lying/slatted dunging, c= solid (scrape-through dung), d= solid (straw bedded)
-stocking of buildings, a= all-in/all-out, b= continuous throughput
-washing/disinfection between batches, a= always done, b= variably done, c= seldom done

For study 2, each farm was given a coding reference number. The information of farms for study 2 was collected as follows:

1. General farm information:

-name of producer, farm address, vet practice
-farm size: number of sows 1=<100, 2= 100-200, 3= 201-400, 4= 401-600, 5= >600

2. Herd health:

-breeding stock replacement as gilt source, boar source
-herd health status of each farm was based on clinical history and laboratory investigations carried out as routine basis previous to the infectious colitis sampling, and was coded as (1= positive, 0= negative) for each of the following diseases: *Streptococcus suis*, atrophic rhinitis, swine dysentery, enzootic pneumonia, proliferative enteropathy, *Actinobacillus pleuropneumoniae*, porcine reproductive and respiratory syndrome (PRRS), sarcoptic mange and overall health status.

3. Sample group:

-faecal samples taken (1= yes, 0= no), number of faecal samples taken, number of pigs taken for postmortem examination, weight of pigs at postmortem, weight of pigs at first diarrhoea.

4. Pre-weaning (0-21 days):

-feeding information coded as (1= yes, 0= no) for creep offered to piglets, creep sample, feed medicated, feed offered, growth promoter (GP, allowing 3 entries GP1-GP3)

-days at weaning

5. Housing 1 post-weaning (21-60 days):

-housing information coded as (1= yes, 0= no) for pens all-in/all-out, buildings all-in/all-out, cleaning between batches, disinfection, and disinfectant used

-days in pen, average weight in/weight out (kg), stock density, number of pigs in pen, pen size (m^2), pen hygiene coded as (0= very clean to 3= extremely dirty), floor type (B= bare, FS= fully slatted, PS= partially slatted, ST= straw, SW= sawdust), ventilation (N= natural, F= fan, ACV= automatically controlled ventilation), type of drinker (N= nipple, T= trough), type of feeder (G= floor, H= hopper, T= trough)

6. Housing 2 post-weaning (21-60 days):

-same as above

7. Feeding history of post-weaning (21-60 days):

-feed offered coded as (AD= *ad libitum*, R= restricted, TA= to appetite), feed type (CR= compound ration, L= liquid, HM= home mix), type of feed (M= meal, P= pellet)

-feeding information coded as (1= yes, 0= no) for feed medicated, enzyme, zinc oxide (ZO), growth promoter (GP), GP-salinomysin (GP-sal), antibiotic (AB) allowing 3 entries AB1-AB3, and feed sample taken

-four feed types allowing four entries F1-F4

8. Housing 1 grower (60-100 days):

-same as point 5

9. Housing 2 grower (60-100 days):

-same as point 5

10. Feeding history grower (60-100 days):

-same as point 7

-four types of feed (F1-F4)

11. Current housing of sample group:

-days since housing changed to the onset of diarrhoea, days in pen, average weight in/weight out, stocking density, number of pigs in pen, pen size (m^2), pen hygiene, type of floor, type of ventilation

12. Current feeding of sample group:

-days since diet changed to the onset of diarrhoea, feed offered, feed type, days on feed, feed type, type of feed

-feeding information coded as (1= yes, 0= no) for feed medicated, enzyme, ZO, GP, GP-Sal, AB and feed sample taken

The sample group represent the stage at which pigs were affected with diarrhoea in the IC and NSC farms, and the equivalent age of pigs in control farms (not affected with diarrhoea).

Analysis

Study 1

As the data in study 1 comprised a limited number of variables and these were also included in study 2, the data from both studies were merged as describe below.

Study 2

The variables of management and feeding systems were divided into the following classes:

-age of pigs (mean days±SE): pre-weaning, up to 24.6±0.5; post-wean, up to 61.5±1.1; grower, up to 82.5±2.6, this was done to standardise the data for any differences in age of pigs.
-food/accommodation: a category called "current" was assigned to the conditions (food and accommodation) the pigs were exposed to at the time of sampling. There were a large number of variables generated in this study. For example, post-weaning pigs were fed up to 4 different diets and had as many as 2 accommodations. In an attempt to decrease the number of variables and provide more meaningful comparisons, the data was averaged across stages of development. So, summary variables such as "at some point" or "mixture" were created for instances where different feeds or types of medications were used within a stage of development.
-management/accommodation: variables such as type of ventilation, type of drinker, level of hygiene, gilt and boar source, presence of any infectious diseases present in the farm at the time of sampling and food given to preweaning pigs. Some of these variables, particularly feeding system, flooring and drinker type were categorised as mixture (explained above) since farms had more than one type of those variables at the different stages; for instance, farms could have the two, nipple or trough drinkers at the same time. So those variables were analysed as combined.

In order to increase sample size in study 2, farms of the category NSC were merged with control farms since there were no infectious agents involved. Thus, for categorical data the significance of variables was tested on comparison between control farms (n=27) and IC farms (n=20) farms by odds ratios using χ^2 test and taking the Fisher's Exact test values. Then 90 % confidence intervals (CI) were calculated.
For continues variables the analysis was performed by analysis of variance (ANOVA). Significance was set at a probability of $p \leq 0.10$.

Data was analysed in a univariate analysis in which categorical and continuous variables were included, and in a multivariable analysis in which only categorical variables were included. These two modalities were set at a significance level of $p \leq 0.10$. Then, all the

variables from those screening analyses showing significance at the level of $p \leq 0.250$ were entered into a logistic regression analysis. In order to increase numbers in the analysis, farms from study 2 with the variables: source of replacements (bought in single source or various sources, or home bred), type of feed (pellet or meal), type of floor (fully slatted as solid lying/slatted dunging or straw bedded), type of management system for batches of pigs (all-in/all-out or continuous), and washing and disinfection between batches (always done or variably done) were merged with farms of study 1. All the analyses of data were performed on SAS statistical computer package (version 8.0).

Odds ratio ψ is defined as the ratio of the probability of an event occurring to the probability of it not occurring (a ratio little used out side betting circles). By contingency tables the statistical significance of factors associated with infectious colitis were established using χ^2 test. In a case-control study the OR, ψ_e, is the ratio of the odds of exposure to the hypothetical (risk) factor in diseased animals (cases) to the odds of the exposure in non-diseased animals (controls).

	Diseased	**No disease**	**Total**
Exposed	a	b	e
Non-exposed	c	d	f
Total	g	h	n

The calculation of odds ratio in a case-control study,

$$\text{Exposure odds}_{\text{cases}} = a/b$$

$$\text{Exposure odds}_{\text{controls}} = b/d$$

$$\text{Exposure odds ratio } (\psi_e) = (a/b)/(c/d) = ac/bd$$

An OR greater than 1 indicates an association between the hypothetical factor and the disease. The greater the OR the higher the statistical association of the hypothetical factor with the incidence of disease in exposed individuals than in unexposed individuals. Therefore an OR of 2 is interpreted as twice the incidence of the disease in the exposed animals. An OR less than 1 indicates a negative statistical association of the hypothetical factor with the disease incidence and has a protective effect against the disease. An OR of 1 indicates no association of the factor with the disease incidence.

The calculation of confidence intervals (CI) provides statistical significance. That level of significance could be set at 80 %, 90 %, 95 %, 99 % or 99.9 %. In risk factor analysis, if the CI includes 1, then the OR is not statistically significant, whereas if the CI does not include 1 implies statistical significance. The following is a worked example of enzootic pneumonia (Thursfield, 1995), in which an OR = 2.99 for fan ventilation indicates an association between that factor and higher incidence of that disease in exposed herds. To test the statistical significance of that OR value a 95 % CI is calculated, which for that example is (1.71, 5.23). Since the 95 % CI does not include 1 in its range, the factor (fan ventilation) is said to be associated with higher incidence of enzootic pneumonia in the exposed animals at that level of significance ($P<0.05$) (Thrusfield, 1995).

Results

Multivariate analysis.

The variables flooring type: fully slatted or straw bedding; type of feed: meal or pellet; source of breeding stock replacements: single or multiple; production system: all-in/all-out or continuous, and washing of premises: always or variably done were the same for both data sets (study 1 and study 2); therefore they were merged to increase sample size. Interestingly, the flooring type straw bedded was significantly associated with occurrence of porcine infectious colitis ($p<0.007$), whereas the flooring type slatted floors was found to be protective ($p<0.007$) (Table 2.1).

Table. 2.1. Variables for both data sets studies (study 1 and study 2 merged) and their association with porcine infectious colitis ($p<0.10$).

Merged variables	IC farms	C farms	Odd ratios (90 % CI)*	P value
Flooring type				
fully slatted	13	28	0.28 (0.13-0.62)	***0.007***
bedding	23	14	3.5 (1.6-7.8)	***0.007***
Type of feed				
meal	28	37	0.71 (0.40-1.26)	0.33
pellet	36	34	1.40 (0.79-2.47)	0.33
Source of breeding stock				
single	51	57	0.50 (0.19-1.31)	0.22
multiple	9	5	2.01 (0.76-5.30)	0.22
System				
all-in/all-out	13	16	0.88 (0.44-1.75)	0.75
continuous	51	55	1.14 (0.57-2.28)	0.75
Washing of premises				
always done	19	33	1.47 (0.81-2.66)	0.28
variably done	45	38	2.06 (1.30-3.70)	0.28

P values *in italics* and bold were significant

* Fisher's exact test

The multivariate analysis by logistic regression confirmed the significant association of the variables, source of gilt replacements from breeding company A ($p<0.03$) and the presence of *S.suis* in the farm with infectious colitis ($p<0.04$) (Table 2.2).

Table 2.2. Categorical variables analysed by logistic regression for association with porcine infectious colitis ($p<0.05$).

Variable	Odds ratio (95 % CI)	P value
Ventilation	1.4 (0.38-5.16)	0.61
Type of drinker	0.63 (0.206-1.96)	0.43
Hygiene	2.79 (0.42-18.37)	0.28
Gilt source (from farm A)	5.83 (1.10-30.68)	***0.03***
Respiratory disease	1.77 (0.305-10.26)	0.52
S. suis	5.72 (1.04-31.43)	***0.04***
Feed given preweaning	0.26 (0.04-1.52)	0.13

P values *in italics* and bold were significant

Study 2

As NSC farms were merged with control farms the total number of control farms was 27 and the total number of IC farms was 20. For the farm size variable of the IC farms 25.0 % (5/20) had <200 sows, 40.0 % (8/20) had 200-400 sows and 35.0 % (7/20) had >400 sows. For the control farms 25.9 % (7/27) had <200 sows, 48.1 % (13/27) had 200-400 sows and 25.9 % (7/27) had >400 sows. As for the variable single or multiple origin of the breeding replacements 95.0 % (19/20) of IC farms and 92.5% (25/27) of the control farms had replacements from a single source. Regarding the variable type of farms (open i.e. breeding stock brought in to the farm from another source, or closed i.e. no breeding stock brought in to the farm from another source), 100 % of the farms were open. Of the breeding stock sources (gilts and boars), up to 6 commercial breeding companies were recorded and designated as (A, B, C, D, E and F). There was a high proportion of farms getting breeding stock replacements (gilts and boars) from a company A. Of the IC farms, 60.0 % (12/20) had gilts and boars from the breeding

company A followed by 15.0 % (3/20) of boars from the breeding company C. Of the control farms, the proportions were more distributed as 25.9 % (7/27) had gilts from two breeding companies A and B, and 37 % (10/27) of the farms obtained boars from company A followed by 22.2 % (6/27) of the farms obtaining boars from two different companies B and C. The proportion of farms having at least one infectious disease other than colitis at the time of sampling was 70.5 % (12/17) for the IC farms and 58.3 % (14/24) for the control farms. In relation to particular endemic infections, IC farms were more affected than control farms, for instance, 52.6 % (10/19) of IC farms were affected by *S. suis* infection compared to 26.9 % (7/26) of control farms and 63.1 % (12/20) of IC farms had PRRS compared to 38.4 % (12/26) of the control farms (Table 2.3).

Table 2.3. Proportion of farms for the variables of -characteristics of farm-, absolute numbers are in parenthesis.

Variable	IC Farms (20)	*Control farms (27)*
Farm Size:		
< 200	25.0 % (5)	25.9 % (7)
200 – 400	40.0 % (8)	48.1 % (13)
> 400	35.0 % (7)	25.9 % (7)
Source of Pigs:		
single source	95.0 % (19)	92.5 % (25)
multiple source	5.0 % (1)	7.5 % (2)
Breeding system:		
Open	100 % (20)	100 % (27)
Closed	0	0
Gilt source:		
A	60.0 % (12)	25.9 % (7)
B	10.0 % (2)	25.9 % (7)
C	10.0 % (2)	22.2 % (6)
D	0	7.4 % (2)
E	0	0
Home	5.0 % (1)	7.4 % (2)
Mix	5.0 % (1)	0
Other	10.0 % (2)	11.1 % (3)
Boar source:		
A	60.0 % (12)	37 % (10)
B	10.0 % (2)	22.2 % (6)
C	15.0 % (3)	22.2 % (6)
D	5.0 % (1)	7.4 % (2)
E	0	0
Home	0	0
Mix	0	0
Other	10.0 % (2)	11.1 % (3)
Previous Health Problems:		
at least 1*	70.5 % (12) (n=17)	58.3 % (16) (n=24)
Health status:		
S.suis	52.6 % (10) (n=19)	26.9 % (7) (n=26)
Atrophic rhinitis	52.6 % (10) (n=19)	0 (0) (n=27)
Swine dysentery	52.6 % (10) (n=19)	0 (0) (n=27)
Proliferarive enteropathy	60.0 % (12) (n=20)	46.1 % (12) (n=26)
A. pleuropneumoniae	27.7 % (5) (n=18)	3.4 % (1) (n=26)
PRRS	63.1 % (12) (n=19)	38.4 % (10) (n=26)
Sarcoptic mange	5.0 % (1) (n=20)	7.7 % (2) (n=26)

Concerning accommodation for the current group for the variable number of pigs/pen, the proportion of farms from both IC and control farms was quite similar since 50.0 % (10/20) of the IC farms and 44.4 % (12/27) of the control farms had >50 pigs/pen. Interestingly, a high proportion 70.0 % (14/20) of IC farms were regarded as "dirty" whereas 55.5 % (15/27) of the control farms were classified as "clean". For the variable pens all-in/all-out, all the IC and control farms had this system for postweaning pigs but only 50.0 % of the IC farms and 59.3 % of control farms practiced this system for grower pigs. A low proportion of both IC and control farms practiced all-in/all-out for buildings for postweaning pigs and an even lower proportion was observed for grower pigs in both cases. As for the type of flooring, 55.5 % (15/27) of the control farms had fully slatted floors compared to 36.8 % (7/20) of IC farms, and 47.3 % (9/19) of IC farms had solid floors with bedding material as compared to 25.9 % (7/27) of control farms. For the type of drinker a high proportion (81.4 %) of the control farms had nipple drinkers as compared to 55.0 % (11/20) of IC farms. The proportion of farms for the type of feeder was very similar as 45.0 % (9/20) of IC farms and 15.5 % (15/27) of the control farms had hopper feeder (Table 2.4).

Table 2.4. Proportion of IC and control farms for the variables of –housing/ accommodation-, absolute numbers are in parenthesis.

Variable	IC Farms (20)	*Control farms (27)*
No. pigs/pen		
<25	20.0 % (4)	33.3 % (9)
25-50	30.0 % (6)	22.2 % (6)
>50	50.0 % (10)	44.4 % (12)
Hygiene status		
clean	30.0 % (6)	55.5 % (15)
dirty	70.0 % (14)	45.0 % (12)
Pens all-in/all-out:		
post wean	100 % (20)	100 % (27)
growers	50.0 % (10)	59.3 % (16)
Builds all-in/all-out		
post wean	25.0 % (5)	22.2 % (6)
growers	5.0 % (1)	3.7 % (1)
Flooring system		
fully slatted	36.8 % (7)	55.5 % (15)
straw bedded/saw dust	47.3 % (9)	25.9 % (7)
partially slatted	15.7 % (3)	18.5 % (5)
Ventilation		
none	60.0 %(12)	29.6 % (8)
fan	25.0 % (5)	59.2 % (16)
air conditioning	15.0 % (3)	11.1 % (3)
Type of drinker		
nipple	55.0 % (11)	81.4 % (22)
nipple + trough	10.0 % (2)	3.7 % (1)
trough	35.0 % (7)	14.8 % (4)
Type of feeder		
hopper	45.0 % (9)	15.5 % (15)
trough	25.0 % (5)	14.8 % (4)
hopper + trough	20.0 % (4)	25.9 % (7)
floor	10.0 % (2)	3.7 % (1)

Regarding feeding system for the "current" category, 65.0 % (13/20) of the IC farms and 63.0 % of the control farms (17/27) had the feeding as *ad libitum*. For the variable feed type, a higher proportion 62.9 % (17/27) of control farms offered the feed as meal type compared to 55.0 % (11/20) of IC farms. As for the variable feed medicated, 60.0 % (12/20) of the IC farms had the feed medicated compared to 44.5 % (12/27) of control

farms. Interestingly, none of the IC farms included enzyme in the feed but 35.0 % (7/20) of those farms included a growth promoter. As for the control farms, 7.4 % (2/27) of the farms included enzyme and 25.9 % (7/27) included growth promoter in the feed (Table 2.5).

Table 2.5. Proportion of IC and C farms for the variables of -feeding system-, absolute numbers are in parenthesis.

Variable	IC Farms (20)	*Control farms (27)*
Feed offered		
to appetite	35.0 % (7)	37.0 % (10)
ad libitum	65.0 % (13)	63.0 % (17)
Feed type		
compound ration	45.0 % (9)	33.3 % (9)
home mixed	45.0 % (9)	48.1 % (13)
liquid	10.0 % (2)	18.5 % (5)
meal	55.0 % (11)	62.9 % (17)
pellet	45.0 % (9)	37.1 % (10)
Feed medicated		
no	40.0 % (8)	55.5 % (15)
yes	60.0 % (12)	44.5 % (12)
Enzyme		
no	100 % (20)	92.5 % (25)
yes	0	7.4 % (2)
Zinc Oxide		
no	95.0 % (19)	92.5 % (25)
yes	5.0 % (1)	7.4 % (2)
Growth promoter		
no	65.0 % (13)	74.1 % (20)
yes	35.0 % (7)	25.9 % (7)
GP-sal		
no	85.0 % (17)	96.2 % (26)
yes	15.0 % (3)	3.7 % (1)
Antibiotic		
no	65.0 % (13)	66.6 % (18)
yes	35.0 % (7)	33.3 % (9)

The number of pigs per pen and density of pigs were not significantly different for the comparison between IC and control farms. The days since change of diet to the onset of

diarrhoea for the IC farms were shorter (18.1 ± 2.18 days) than those of the control farms (24.3 ± 2.14 days), and it was significant ($p<0.056$) (Table 2.6).

Table 2.6. Continuous variables (mean ±SE) tested by ANOVA for associations with porcine infectious colitis.

Variable	IC (n=20)	Control (n=27)	P value
Age at weaning	25.3 (0.54)	24.6 (0.51)	0.352
Days at weaning	6.6 (0.31)	6.5 (0.24)	0.789
Number of pigs per pen	67.2 (11.3)	56.1 (9.6)	0.456
Size of pens (m^2)	47.3 (11.1)	42.8 (14.8)	0.822
Density	1.91 (1.51)	2.0 (0.13)	0.601
Days since change of diet to the onset of diarrhoea	18.1 (2.18)	24.3 (2.14)	***0.056***
Days since change of accommodation to the onset of diarrhoea	22.4 (3.10)	19.5 (2.16)	0.435

P value in *italics* and bold was significant

Of the categorical variables, 14 were indicated using a screening step with a significance level of $p<0.250$. Three variables showed significant association with infectious colitis, these were, breeding replacements (gilts) in particular from the breeding company A, flooring (solid floors) and drinker (mixture) ($p<0.05$). In contrast the type of feeder (hopper) was identified as protective ($p<0.001$) (Table 2.5). Another six variables including presence of pathogens such as *S. suis, A. pleuropneumoniae*, the porcine reproductive and respiratory syndrome-PRRS virus, the feeding system (mixture, appetite/*ad libitum*), growth promoter given at some point and ventilation (natural) were significant at the level of ($p<0.10$) (Table 2.7). The variables: presence of respiratory diseases, feed offered preweaning, dirty pens in and type of drinker/trough were approaching significance (Table 2.7).

Table 2.7. Categorical variables (screened at p<0.250) tested by odds ratios (Fisher's exact test) for association with porcine infectious colitis (p≤0.10)*.

Variable	IC (n=20)	Control (n=27)	Odds ratios (90 % CI)*	P value
Farm Characteristics				
gilt source (breeding company A)	12	7	4.3 (1.5-12.1)	***0.03***
Herd health				
S. suis	11	7	3.5 (1.2-9.8)	***0.06***
App	5	1	8.7 (1.1-56.7)	***0.07***
PRRS	13	10	3.2 (1.1-8.7)	***0.08***
Respiratory diseases	15	14	2.8 (0.97-8.04)	0.13
Preweaning				
feed offered	7	15	0.43 (0.16-1.17)	0.23
Postweaning				
feeding system (mixture TA/AL)	6	2	5.4 (1.3-22.9)	***0.05***
growth promoter given ASP	12	9	3.0 (1.1-8.2)	***0.08***
Flooring (mixture)	6	1	11.1 (1.7-71.5)	***0.03***
Drinker (mixture)	4	0	15 (1.2-184)	***0.03***
type of feeder (hopper)	0	19	0.01 (0.001-0.12)	***0.001***
Current condition				
dirty pens	14	12	2.9 (1.05-8.13)	0.13
ventilation/natural	12	8	3.6 (1.3-9.9)	***0.07***
drinker/trough	7	4	3.1 (0.95-10.1)	0.16

P values *in italics* and bold were significant
* Fisher's exact test
TA= to appetite
AL= *ad libitum*
ASP= at some point

Laboratory and postmortem investigations for study 2

Of the IC farms 95 % (19/20) had *Brachyspira* spp, 50.0 % (10/20) had *L. intracellularis* and 45.0 % (9/20) had *Yersinia* spp. Parasites were the less frequent cause of colitis as 5.0 % (1/20) of the IC farms had Strongyles (Table 2.8). Among the *Brachyspira* spp. the most prevalent was *B. pilosicoli* since it was found in 40.0 % (24/60) and 29.5 % (54/183) of the postmortem and faecal samples, respectively. The frequency of isolation of *B. hyodysenteriae* was at the level of 16.6 % (10/60) and 11.1 % (20/180) from PM examinations and faecal samples, respectively, whereas the frequency of isolation of *B. innocens* and *B. intermedia* was lower than 5.0 %. Interestingly, two isolates of atypical

Brachyspira spp. from PM examinations (3.3 %) and faecal samples (1.1 %) were recovered from IC farms. Both *Y. enterocolitica* and *Y. paratuberculosis* had the same prevalence in faecal samples which was also similar to that of *B. hyodysenteriae*. Also interesting, the prevalence of *L. intracellularis* in faecal samples was very similar to that of *B. pilosicoli* (Table 2.9).

Table 2.8. Proportion of IC farms (20 farms) in which a pathological agent was isolated or identified by PCR.

Pathological agent	Number of farms	Percentage
Salmonella spp.	2	10.0
Yersinia spp.	9	45.0
Brachyspira spp.	19	95.0
Strongyle	1	5.0
*Lawsonia intracellularis**	10	50.0

* identified by PCR

Table 2.9. Pathological agents cultured or identified by PCR from postmortem examinations (3 pigs/farm n= 60) or faecal samples (9 samples/farm n= 180) taken from IC farms.

Pathological agent	From PM examination	Percentage	From faecal samples	Percentage
S. typhimurium	0	0.0	2	1.1
S. thompson	1	1.6	0	0.0
Y. enterocolitica	5	8.3	21	11.6
Y. pseudotuberculosis	7	11.6	20	11.1
B. hyodysenteriae	10	16.6	20	11.1
B. pilosicoli	24	40.0	54	29.5*
B. innocens	0	0.0	3	1.6
B. intermedia	3	5.0	5	2.7
B. murdochii	0	0.0	0	0.0
Atypical *Brachyspira* spp	2	3.3	2	1.1
L. intracellularis	14	23.3	17	28.3**
Strongyle	1	1.6	7	3.8

* n=183

** n=60

PM= postmortem

B. innocens, B. intermedia and *B. murdochii* were also isolated from control farms, the frequencies of isolation from PM examinations were at the level of 17.2 % (14/81), 6.1 % (5/81) and 2.4 % (2/81), respectively; and from faecal samples 5.7 % (14/243), 6.1 % (15/243) and 0.41 % (1/243), respectively (Table 2.10).

Table 2.10. *Brachyspira* spp. isolates from PM examinations (3 pigs per farm n= 81) and faecal samples (9 samples per farm n= 243) taken from control farms, percentages are in parenthesis.

Isolate	*From PM examinations*	From faecal samples
B. hyodysenteriae	0 (0.0 %)	0 (0.0 %)
B. pilosicoli	0 (0.0 %)	0 (0.0%)
B. innocens	14 (17.2 %)	14 (5.7 %)
B. intermedia	5 (6.1 %)	15 (6.1 %)
B. murdochii	2 (2.4 %)	1 (0.41 %)

B. pilosicoli was found as a single pathogen in 20.0 % (4/20) of the IC farms and 40.0 % (8/20) in conjunction with other pathogens. The frequency of finding *B. pilosicoli* together with *L. intracellularis* and other pathogen was at the level of 25.0 % (5/20). The prevalence of *B. hyodysenteriae* as a single pathogen was lower (5.0 % 1/20) than that of *B. pilosicoli*, however, the prevalence of *B. hyodysenteriae* together with *L intracellularis* and other pathogens was at the level of 20.0 % (4/20) which was fairly similar to that of *B. pilosicoli* together with *L. intracellularis* and other pathogens. Of the 20 IC farms, 60.0 % (12/20) had involved a *Brachyspira* spp in co-infection with another pathogen (Table 2.11).

Table 2.11. Proportion of IC farms in which *Brachyspira* spp were identified as cause of colitis.

Pathological agent	No. farms	Proportion
B. pilosicoli	4	20.0 %
B. pilosicoli + *L. intracellularis*	1	5.0 %
B. pilosicoli + *L. intracellularis* + other pathogen	5	25.0 %
B. pilosicoli + other pathogen	8	40.0 %
B. hyodysenteriae	1	5.0 %
B. hyodysenteriae + *L. intracellularis*	1	5.0 %
B. hyodysenteriae + *L. intracellularis* + other pathogen	4	20.0 %
B. hyodysenteriae + other pathogen	7	35.0 %
Other pathogen	1	5.0 %
Brachyspira spp + other pathogen	12	60.0 %

Other= *Salmonella* spp., *Yersinia* spp., or parasites.

Discussion

This study analysed variables thought to be associated with porcine infectious colitis. Some of those variables were the same for two different data sets (study 1 and study 2) collected 3 years apart. As sample size is always important in statistical analysis, merging data of same variables from the two studies increased statistical power. The epidemiology of PCS has not been fully determined yet so, identification of possible risk factors associated with this disease would help to understand this disease better.

The study showed that the variables breeding replacements (gilts) in particular from the breeding company A, flooring (solid floors) and drinker (mixture) were significantly associated with the onset of infectious colitis ($p<0.05$). Other variables such as presence of pathogens such as *S. suis, A. pleuropneumoniae*, the porcine reproductive and respiratory syndrome-PRRS virus, the feeding system (mixture to appetite/*ad libitum*, growth promoter given at some point and ventilation (natural) were also significantly associated with infectious colitis ($p<0.10$). On the contrary, the variables slatted floors and the type of feeder 'hopper' were found to be protective.

In this study, gilt source (breeding company A) was identified as a factor associated with infectious colitis. The identification of gilt source as a factor associated with infectious colitis support information reported in the literature regarding the transmission of SD and PCS by carrier animals, for instance, epidemiological studies have found associations of gilt source with enteric diseases, particularly, SD (Robertson *et al,* 1992) and proliferative enteropathy (PE) (Smith, 1997). In the latter study, nucleus herds were infected with *L. intracellularis* and therefore were the source of infection for commercial farms (Smith, 1997). Since *B. pilosicoli* or *B. hyodysenteriae* were found in mixed infections together with *L. intracellularis* and other pathogens at levels of 25.0 % (5/20) and 20.0 % (4/20), respectively in this study, it is possible that source of breeding replacements was a key factor for both diseases. Hence detection of carrier animals before they are introduced into susceptible farms is important in order to reduce risk of

disease transmission. Other epidemiological studies have also identified an association between source of breeding replacements or bought-in pigs with infectious enteric diseases. For instance, epidemiological tracing of *B. hyodysenteriae* outbreaks identified that the source of infection of 22 out of 25 outbreaks was by carrier pigs purchased from previously infected nucleus farms (Windsor and Simmons, 1981). Interestingly, in that study according to records the period between the entrance of *B. hyodysenteriae* in a farm and the appearance of the clinical signs could be as long as three years, raising questions about the survival of this pathogen for long periods without clinical manifestation, and also what triggers the clinical manifestation after being apparently latent. In another study it was shown that *L. intracellularis* infection could be transmitted to a new batch of pigs despite 10 months interval since the last evidence of clinical disease, as the new pigs were introduced into pens that had previously housed pigs affected with PE (Smith, 1997). Those results indicated that *L. intracellularis* remained latent in that herd where an outbreak of PE had occurred and latency was also confirmed by the follow-up of an acute outbreak in which PCR positive samples were detected after five months of the occurrence of the outbreak (Smith, 1997). Interestingly, in that study all the samples taken from suckling piglets were PCR negative, suggesting that *L. intracellularis* infection may not be present in young pigs (Smith, 1997). However, as for porcine intestinal spirochaetes it has been reported the isolation of *B. hyodysenteriae* and *B. pilosicoli* in suckling pigs of 3 to 5 weeks of age from herds with and without clinical diarrhoea (Fellström *et al,* 2001).

The transmission of disease to the receiving farm will depend on factors such as herd immunity, and the probability of the receiving farm to develop the disease will depend on the amount of microbes transmitted (Madec *et al,* 1993). A study evaluated the health and transmission of diseases by nucleus herds into commercial herds by measuring the clinical effect and lesions on specific pathogen free (SPF) piglets that were put in contact with replacement gilts from nucleus herds. In that experiment, SPF piglets showed minimal or no clinical signs or lesions after being in contact with gilts from top health nucleus herds, whereas SPF piglets in contact with gilts from nucleus

herds with known diseases developed those diseases. Those results confirmed disease transmission by breeding stock replacements (Madec *et al,* 1993). It has also been reported that faecal excretion of *B. hyodysenteriae* can be suppressed by antimicrobials given in the feed but it does not eliminate transmission (Griffin and Hutchings, 1980). Interestingly, in the present study the use of growth promoter in feed emerged as a significant factor for infectious colitis. The study by Smith (1997), identified as significant the difference of between affected 94.0 % (15/16) and non-affected herds 58.0 % (24/41) by PE when using chlortetracycline on "occasionally" basis. In another study, *B. pilosicoli* infections became recurrent despite treatment with lincomycin or tiamulin in herds that did not give prophylactic medication (Thomson *et al,* 1998).

In agreement with previous reports (Robertson *et al,* 1992; Smith, 1997), the present study found that the source of gilt replacement (breeding company A) was significantly associated with infectious colitis. Interestingly, that particular company supplied gilts to 60.0 % (12/20) of the IC farms and to 37.0 % (10/27) of the control farms. Surprisingly, the variable boar source was not associated with infectious colitis despite the same breeding company supplying boars to the farms. A possible explanation could be that boars may be housed in individual pens and do not have the same degree of contact with other pigs in the farm as brought-in gilts. They may also be kept in isolation for artificial insemination purposes. If that was the case, the risk of transmission of *B. pilosicoli, B. hyodysenteriae* or an intestinal pathogen could be very low if no contact between animals occurs and strict hygiene measures were applied. However, transmission of infectious agents could be through human contacts or vectors such as domestic animals or rodents (Alexander *et al,* 1980).

In another epidemiological study in Western Australia found that visitors allowed into farms was approaching significance as a risk factor for the occurrence of SD ($p<0.08$) since farms allowing visitors were 3.3 times more at risk (Robertson *et al,* 1992). On the contrary, overalls and rubber boots provided to visitors was regarded a protective factor as the OR was at the level of (0.22) ($p<0.04$) (Robertson *et al,* 1992). Reducing the risk

of introduction of diseases into pig operations could be done by biosecurity measures such as security fencing, overalls and rubber boots provided to visitors, showers taken before and after entry to a farm. All these measures play an important role in preventive medicine that includes cleaning and disinfection which has been identified as protective for the occurrence of enteric diseases in pigs (Pearce, 1999). The efficacy of disinfectants on field isolates of *B. pilosicoli* was assessed as a control measure in Chapter 5.

In this study the presence of certain endemic infectious agents on IC farms at the time of sampling was associated with infectious colitis, in particular, the presence of *A. pleuropneumoniae* and PRRS virus. The reason for this is uncertain but it is possible that such endemic infections compromise the immune system allowing enteric pathogens to become established more readily.

Respiratory diseases in pigs have also been reported as co-infections, in particular, between respiratory viruses and bacteria, for instance, PRRS virus and *Mycoplasma hyopneumoniae* (Zeman *et al,* 1993). In this co-infection the virus may predispose pigs to bacterial infections since this virus persistently damages macrophages (Molitor, 1994). Another association of pathogens affecting pigs is between AD virus and *A. pleuropneumoniae*, in which the risk of Aujeszky's disease virus being circulating in pig herds has been reported as 2 times higher if *A. pleuropneumoniae* is present in the herd (Anderson *et al,* 1990). So, association of pig pathogens in field conditions is frequent; however, sometimes experimental models have not been able to demonstrate those associations which suggests that the clinical expression of certain pathogens could be complex and some environmental factors may be playing a role for disease manifestation. For instance, pigs intranasally inoculated with PRRS virus at 3 weeks of age followed by intratracheal inoculation with *M. hyopneumoniae* one week after did not develop the characteristic pneumonia seen in field cases when these two pathogens co-exist in pig herds (Alstine *et al,* 1996).

The fact that PRRS virus causes immunosuppression could explain its association with porcine colitis since secondary bacterial infections could develop in immunosuppressed individuals due to damage to macrophages caused by this particular virus. Interestingly, in humans intestinal spirochaetal infections have been reported associated with immonocompromised individuals by the human immonodefficiency virus (HIV) which also kills macrophages (Law *et al,* 1994), thus individuals affected by HIV become susceptible to secondary bacterial infections such as intestinal spirochaetosis.
Mixed enteric infections were also identified in the current study and could have further compromised the pig immune response to infections.

Yersinia pseudotuberculosis has also been confirmed as an aetiological agent of colitis in pigs as the disease was experimentally reproduced (Neef and Lysons, 1994). In that study, *Y. pseudotuberculosis* was isolated from 3 out of 7 pigs farms where *Salmonella* spp. was not recovered on culture, and *B. hyodysenteriae* had not been identified. However, it was suggested that *Y. pseudotuberculosis* might act in conjunction with other pathogens which could enhance its clinical manifestation in porcine infectious colitis (Neef and Lysons, 1994).

In this study, the presence of *S. suis* was significantly associated with infectious colitis ($p<0.04$). This result was unexpected as one could think that there is no links between the two pathogens in terms of pathogenic tropism. Interestingly, in an epidemiological study in England the presence of *S. suis* causing meningitis was at the level of 57.0 % (60/105) which was a higher proportion than that of PRRS (31.0 %, 33/105) (Pearce, 1999). Although in the present study no laboratory investigations were attempted for the identification of *S. suis* due to the nature of this disease which is localised in the brain and characterised by meningitis, and may be implicated in bronchopneumonia in cases associated with *M. hyopneumoniae* (Taylor, 1995). Further studies need to be done to investigate more deeply the association of *S. suis* with porcine colitis.

In this study, 5.0 % (1/20) of the IC farms had mixed infections with *B. pilosicoli* and *L. intracellularis*, and 25.0 % (5/20) had a mixed infection with *B. pilosicoli, L. intracellularis* and another pathogen. Similar proportion of IC farms had mixed infection with *B. hyodysenteriae* and *L. intracellularis*, and 20.0 % (4/20) had mixed infections with *B. hyodysenteriae, L. intracellularis* and other pathogen, 60.0 % (12/60) of the IC farms had mixed infections in which *Brachyspira* spp. was found together with another pathogen. These results are in agreement with other studies where mixed infections with *L. intracellularis* and WBHIS group IV (i.e. *B. pilosicoli*) have been found at the level of 8.0 % (6/72), and with *L. intracellularis* and *B. hyodysenteriae* at the level of 10.0 % (7/72) similar proportion (11.0 %) of herds had mixed infections with *L. intracellularis* and *S. enterica* (Møller *et al,* 1998). Interestingly, in that study herds that had *L. intracellularis* were 1.5 times ($p<0.10$) more at risk to develop diarrhoea, and higher proportion (39.0 %, 28/72) of herds had a mixed infection with *L. intracellularis* and haemolytic *E. coli* than with *L. intracellularis* and WBHIS (29.0 %, 21/72) (Møller, 1998). In the present study isolation of *E. coli* was not undertaken as affected pigs exceeded the age when colibacillosis occurs.

B. innocens, B. intermedia and *B. murdochii* were isolated from PM examinations and faecal samples of pigs from control farms in this study. Interestingly in the study by Møller *et al* (1998), *L. intracellularis* and WBHIS were also isolated (from 3 out of 260 pigs) from control farms. The fact that in the present study *B. intermedia* and *B. murdochii* were isolated from control farms in which diarrhoea was not observed at the time of sampling supports the belief that these organisms are non-pathogenic commensals. However, (Fellström and Gunnarson (1995) suggested that *B. intermedia* may be involved a mild colitis in pigs. Also This could be significantly important for the classification of 'control' as control herds should be such in which absence of any bacterial pathogen is necessary to fulfill the condition of being free of disease when case-control studies are investigated (Møller *et al,* 1998).

The feeding system 'mixture' (TA/AL) was significantly associated ($p<0.05$) with infectious colitis in this study. The reason for this is uncertain. However, in Sweden the use of restricted feeding system in postweaning pigs instead of *ad libitum* coupled with increasing the feed intake of lactating sows and hygiene by training farm personnel was used as an approach to control postweaning diarrhoea (Löfstedt *et al,* 2002). In another study farms with wet feed system were 5.9 more times at risk to develop infectious colitis (Pearce, 1999). In the current study there were not sufficient farms with wet feed to be able to draw any conclusion in relation to this particular factor.

The days since diet changed were significantly associated with infectious colitis in this study. The significance of IC farms with an average of 18.1 days since diet changed compared to an average of 24.3 days for the control farms is unclear. In some instances diet changes coincide with accommodation changes, the latter have been associated with intestinal disorders since onset of diarrhoea could develop 7 to 14 days after mixing or moving pigs (Thomson *et al,* 1998). Although this study did not evaluate the diet composition, it has been suggested that highly fermentable polysaccharides are associated with the occurrence of swine dysentery (Pluskey *et al,* 1998). The manifestation of clinical signs of pathogens such as *B. hyodyseteriae* and *L. intracellularis* may be due to interaction with the indigenous bacterial population in the gut of pigs and perhaps also the composition of diet (Leser *et al,* 2000).

In view of potential transmission of infectious diseases by direct contact with infective material, slatted floors were designed to keep premises cleaner and drier to reduce the risk of faecal contamination. The removal of infective material (e.g. faeces containing intestinal spirochaetes) from pens and buildings is essential to prevent susceptible animals becoming infected by contact with such material and the removal of that material is also important before applying disinfection (Chapter 5). The removal of such material is done manually or mechanically in farms that do not have slatted floors. The frequency of cleaning varies between farms and pigs may be at risk of becoming infected by prolonged or recurrent exposure to infective material. In the present study,

slatted floors were identified to be protective ($p<0.007$) for infectious colitis, whereas solid bedded floors were significantly associated with the disease ($p<0.007$). The use of slatted floors has a double benefit as it provides less labour and improves hygiene. This study suggests that reduction in the risk of infectious colitis is an added benefit. In contrast, in a study to determine the risk factors for porcine proliferative enteropathy (PE) caused by *Lawsonia intracellularis*, slatted floors were identified as a significant risk factor for the occurrence of PE ($p<0.05$), whereas bedded floors were found to be protective (Smith, 1997). However, in a comparative study between the use of slatted floors and solid floors, pigs housed in pens with slatted floors had lower morbidity of diarrhoea and also lower total bacterial counts than pigs allocated in premises with solid floors (Rantzer and Svendsen, 2001). In another study, pens with partially slatted floors were associated with enteric diseases in finishers pigs so, partially slatted flooring systems were 3.6 times greater at risk of developing enteric diseases than fully slatted floors (Pearce, 1999).

The variable ventilation (natural) was approaching significance in this study. The reasons for that are unclear but it could that low temperatures let to discomfort so as a consequence of that pigs may develop diarrhoea.

Nipple drinkers were associated with the occurrence of PE ($p<0.1$) (Smith, 1997). In the current study the type of drinker was also significantly identified as risk factor for porcine colitis. Nipple drinkers are designed to keep pens clean and dry and coupled with slatted flooring may contribute to reduce the exposure of pigs to infective material. Wet pens may predispose to the survival of enteric pathogens as it has been reported that the survival of *B. hyodysenteriae* in manure could be up to 60 days when maintained at low temperature (Bale *et al*, 1993), and *B. hyodysenteriae* contained in lagoon effluent water for as long as 6 days was still able to cause disease in pigs (Olson, 1995).

Out of this epidemiological study, it was observed that infectious colitis is multifactorial disease since more than one factor was significantly associated with it, and more than

one infectious agent may be present in the pathogenesis. Therefore, for evaluation of herd health of pig operations one should be more opened to the possibility of microbe interactions, and the role of the environment has also to be considered.

In conclusion, infectious colitis in pigs is a multifactorial disease in which infectious agents such as *Brachyspira* spp. and *L. intracellularis* are the most prevalent, and may interact with other enteropathogens like *Yersinia* spp. and *Salmonella* spp. to cause infection. Infectious colitis could be influenced by environmental or management factors such as gilt source, bedded floors or presence of other pathogens such as *Streptococcus suis* as could be observed in these epidemiological and bacteriological investigations.

Chapter three

ASSESSMENT OF IMMUNOMAGNETIC SEPARATION (IMS) FOR DETECTION OF INTESTINAL SPIROCHAETES (*Brachyspira pilosicoli* and *B. hyodysenteriae)* IN FAECAL SAMPLES

Introduction

The main diagnostic test for SD and PCS is the isolation of the causal micro-organisms from faecal samples of affected pigs by bacteriological culture on selective media. This is complemented with biochemical tests (Fellström and Gunnarson, 1995; Hommez *et al*, 1998) for speciation, particularly in laboratories lacking in molecular techniques. The detection of porcine intestinal spirochaetes by bacterial culture is more complicated from carrier pigs that are shedding only low numbers of bacteria in faeces after recovering from the disease (Alexander and Taylor, 1969). Carrier pigs are important in the transmission of SD since these animals carry the bacteria without showing clinical signs. Little is known about the role of carriers in PCS particularly but the current reports for SD may apply to PCS since pigs can remain infected with *B. pilosicoli* for up to 10 weeks (Duhamel, 1998).

Because porcine intestinal spirochaetes are fastidious organisms the use of various culture media and incubation conditions have been tried in order to develop the most reliable and efficient diagnostic method for their isolation from faecal samples. Various selective culture media supplemented with a range of additives to improve the isolation have been tried. The additives include antibiotics such as spectinomycin (Songer *et al,* 1976), vancomycin, colistin, rifampicin and spiramycin to inhibit other inhabitants of the gastrointestinal tract.

An initial improvement in the isolation of *B. hyodysenteriae* was achieved by a selective medium based on trypticase soy agar (TSA) supplemented with 10 % sheep blood and 400 μg/ml of spectinomycin (TSA-S400) (Songer *et al,* 1976), but this proved insufficiently sensitive since the initial selective medium was not sensitive enough to isolate *B. hyodysenteriae* from rectal swabs, another selective medium was developed. This medium was based on TSA supplemented with 10 % sheep blood and 25 μg/ml colistin, 25 μg/ml vancomycin and 400 μg/ml spiramycin (TSA-CVS) (Jenkinson and Wingar, 1981). That medium proved to be more effective in reducing significantly the

total faecal anaerobes, giving more chance of isolating *B. hyodysenteriae* as pure culture in the first instance.

Another selective medium is based on TSA supplemented with 5.0 % sterile citrate bovine blood, 5.0 % pig faecal extract, and 200 μg/ml spectinomycin, 6.25 μg/ml colistin, 6.25 μg/ml vancomycin, 25 μg/ml spiramycin and 12.5 μg/ml rifampicin (BJ) (Kunkle and Kinyon, 1988). That selective medium was efficient for the isolation of *B. hyodysenteriae* and *B. innocens* as the normal intestinal flora was completely eliminated in 82.0 % (14/17) of the faecal samples. BJ selective medium also had a higher sensitivity as compared with other selective media such as TSA-S400 and TSA-CVS since 27.5 % more isolates of *B. hyodysenteriae* were detected by this medium (Kunkle and Kinyon, 1988).

A comparative study was carried out on the several culture media developed for the isolation of *B. hyodysenteriae* including TSA-S400, TSA-CVS, BJ, TSA-BJ, agar base 2 supplemented with blood and the antibiotics colistin, vancomycin and spiramycin (BA2-SVC), and BA2-BJ. The isolation rates of *B. hyodysenteriae* from faecal samples were best for the BJ medium 89.7 % (130/145) followed by TSA-BJ 88.3 % (128/145), TSA-CVS and BA2-BJ both 76.6 % (111/145), BA2-CVS 75.2 % (109/145), and TSA-S400 52.4 % (76/145) (Achacha and Messier, 1992). BJ was the most efficient in eliminating the normal faecal flora and *B. hyodysenteriae* produced the best growth on this selective medium. Other variables have included slicing the surface of agar (Olson, 1996), and incubation temperatures that range from 37 °C to 42 °C (Jensen, 1997).

Methods that have been developed for the identification of porcine intestinal spirochaete infection include serological tests such as microagglutination and direct or indirect fluorescent antibody test (FAT). The FAT has been applied using polyclonal antibodies raised in rabbit (Burrows and Lemcke, 1981; Lysons, 1991; Hunter and Saunders, 1977) or monoclonal antibodies (MAbs) conjugated with horseradish peroxidase for enzyme labelled immunosorbent assay (ELISA) or indirect immunoflourescence (Achacha and

Mittal, 1996; Lee and Hampson, 1995; Lee and Hampson, 1996). However, due to high cross-reactivity between members of the genus *Brachyspira*, particularly between *B. hyodysenteriae* and *B. innocens*, the specificity of serologic tests has been poor. Attempts to increase the specificity have been made by absorbing antisera with the different species of intestinal spirochaetes but the antibody titre was found to reduce significantly (Lysons and Lemcke, 1983).

Molecular based methods have been developed in recent years, including mainly PCR techniques targeting DNA sequences of the 16S rRNA and 23S rRNA genes of *B. hyodysenteriae* and *B. pilosicoli* (Barcellos *et al,* 2000; Jensen *et al*, 1990; Duhamel *et al*, 1997; Leser *et al*, 1997; Park *et al*, 1995). PCR has been reported to detect as few as 2 X 10^2 of *B. pilosicoli* cells in faecal samples (Mikosza *et al,* 2001). The capability of detecting low numbers by molecular methods is valuable diagnostically for detection of fastidious bacteria that do not grow readily using culture methods, especially when a rapid diagnosis is required.

For improved detection and to provide organisms for epidemiological studies, biotechnological methods are evolving according to diagnostic requirements; hence, new selective techniques have become available. Among these procedures immunomagnetic separation (IMS) for separating target cells from a mixed suspension of cells has been applied increasingly in research and clinical laboratories. The principle of this technique is based on the separation of target cells (eukaryotic or prokaryotic) or proteins by magnetic particles coated with a specific antibody (Ab) that binds those target cells, then magnetic beads are recovered from the mix suspension using a magnet. The first reported uses of IMS were for the isolation of particular cells (B lymphocytes or eosinphils) from whole blood (Rasmussen *et al*, 1990; Blom *et al*, 1995). This procedure has been further developed for separation of bacteria from faeces or food samples. In one study, IMS appeared to be more sensitive than conventional bacterial culture for separating *E. coli* O157 from faecal samples of cattle and sheep

(Heuvelink *et al,* 1998), and from food samples (Wright *et al,* 1994). Similarly, the improved separation of *Salmonella typhimurium* from faecal samples by IMS [magnetic beads coated with monoclonal antibodies (MAbs)] has been achieved (Widjojoamodjo *et al*, 1991). In that study, it was shown that IMS was sensitive enough to detect and separate as few as 100 cfu of *S. typhimurium* from a mixed suspension of bacteria.

A study carried out by Gagné *et al* (1998) demonstrated separation of *Actinobacillus pleuropneumoniae* serotype 1 from tonsils of pigs using magnetic beads coated with purified rabbit immunoglobulin G specific for this serotype. The results of that study showed that IMS was 1000-fold more sensitive than direct culture, detecting also a higher proportion of positive tonsils from infected herds than direct plating for viable bacteria (68.0 % compared to 22.0 %, respectively).

In another study, isolation of *E. coli* O157 from faecal samples was improved by 67.0 % and as many as 12 strains were isolated using IMS compared to only 5 strains detected by direct culture. The efficiency of detecting *E. coli* O157 in asymptomatic patients by IMS was higher than conventional methods, detecting as few as 12 cfu/g of faeces (Chapman and Siddons, 1996). The latter study reported a modified protocol that included a pre-enrichment broth stage using selective media before IMS, which further improved sensitivity as also reported elsewhere (Chapman *et al,* 1997).

Many reports support the improved detection and isolation of target cells by IMS, however, there are other studies that have not found improvement in isolation rates by using IMS to separate those target cells. For instance, Osaki *et al* (1998) reported that the detection rate of *Helicobacter pylori* using IMS and PCR was not higher than that obtained by direct culture.

Further adaptations have combined IMS followed by PCR to successfully increase the detection sensitivity of enteropathogens in faecal samples. Improved sensitivities have been achieved in the detection of *Bacteroides fragilis* (Zhang and Weintraub, 1998),

Salmonella from swine, horses and cattle (Widjojoatmodjo *et al,* 1992; Stone *et al,* 1994), *Helicobacter pylori* (Enroth and Engstrand, 1995), and *Leptospira* spp. from urine samples (Taylor *et al,* 1997). Thus, achieving improved sensitivity in detection of pathogens, particularly fastidious organisms may require a combination of procedures.

Carrier pigs may excrete low numbers of infective bacteria, those low numbers of *B. hyodysenteriae* and *B. pilosicoli* could be transmitted to susceptible animals. To assess the potential of IMS in this regard, a study on the separation of *B. pilosicoli* and *B. hyodysenteriae* from faecal samples was undertaken. Methods for separating these micro-organisms from spiked pig faecal samples were developed and evaluated. The sensitivity of detection by IMS was compared with that of existing diagnostic methods.

Material and Methods

Bacterial isolates

The field isolates that were used for dot blotting, Western blotting and IMS assays were: eight porcine *B. pilosicoli* isolates P32/2/97, P93/2/94, P595/3/00, P657/3/00, P657/8/00, P676/2/00 and P950/3/00; the reference strain for the UK of *B. pilosicoli* (M1); two canine *B. pilosicoli* isolates M1701/99 (K19) and M2901/99 (K22); one human *B. pilosicoli* isolate ExRDVC; the type strain ATCC 51139 of *B. pilosicoli*; six porcine *B. innocens* isolates P692/9/00, P906/3/00, P950/3/00, P1010/2/00, P1015/1A/00 and P105/7B/00; and two *B. hyodysenteriae* isolates P393/8 and P944/14/00. All porcine and canine isolates were obtained from the Scottish Agricultural College-Veterinary Science Division, Edinburgh. The strain M1 was obtained from the Veterinary Laboratory Agency (VLA)-Winchester, England. The human isolate was provided by Dr. Anne Livesley of Aston University, Birmingham, UK.

A variety of bacteria that constitute the normal pig gut flora were also included such as *Lactobacillus acidophilus, Lactobacillus bulgaricus, Clostridium brevis,* total anaerobic bacteria (TAN), mixed enterococci (ENT), total aerobic bacteria (TAE), *E. coli* and mixed coliform bacteria (MC) kindly gifted by Dr. Kevin Hillman (Scottish Agricultural College). Those isolates were used in assays to assess the specificity of monoclonal and polyclonal antibodies.

Monoclonal and polyclonal antibodies

Two monoclonal antibodies (MAbs) IgM subclass produced in mice were gifted by Dr. David Hampson (Murdoch University, Australia). A MAb specific to a 29 kDa envelope protein of *B. pilosicoli* BJL/AC1 (Lee and Hampson, 1995) and a MAb specific to a 30 kDa envelope protein of *B. hyodysenteriae* BJL/SH1 (Lee and Hampson, 1996) were used for coating magnetic beads (M-450) used in IMS assays.

Polyclonal antibody

A polyclonal antibody (PAb) raised in rabbits against *B. hyodysenteriae* was obtained from the VLA-Winchester, England. This PAb has been used in indirect fluorescent antibody test for routine diagnosis of SD at VLA Veterinary Investigation Centres.

Bacterial suspension

Two blood agar (BA) plates per *B. pilosicoli* or *B. hyodysenteriae* isolates were streaked over the whole surface and incubated at 37 °C for 3 days. The bacterial growth was harvested using one sterile cotton swab (Bibby Sterilin, Ltd, UK) per plate then suspended in 1 ml PBS, centrifuged at 8,000 g for 10 minutes and washed twice with PBS. Washed bacterial cells were used as Ag preparations or were 10-fold diluted (10^{1} to 10^{10}) for bacterial cell counting and IMS assays.

Counting of bacterial cells

Counting of viable bacterial cells was carried out for each bacterial suspension used in IMS assays in order to determine the sensitivity of the IMS technique. The drop count method described by Miles and Misra (1938) was the method chosen for viable count of bacterial cells. Briefly, 10-fold dilutions (10^{1} to 10^{10}) of the bacterial suspension were made in 1 ml final volume PBS. The suspension was mixed thoroughly by pipetting 10 times, then 100 µl was transferred to 900 µl diluent to make the first 1:10 dilution. This process was continued to a final dilution of 10^{10}. A 10 µl aliquot of each dilution of the bacterial suspension was inoculated onto BA plates in quadruplicate, then plates incubated anaerobically at 37 °C for 48 to 72 hrs. Colonies from the dilution that produced between 20 and 30 cfu on BA plates were counted and averaged. The total number of cfu/ml were calculated using appropriate dilution and volume multipliers, for example, viable colony counts of quadruplicate inocula from dilution 10^{4} of 21, 23, 28, 27 produce a mean of 24.75 (99÷4). Mutiplication by dilution (10^{4}) and volume ratio of 100 (1000 µl÷10 µl inoculum volume) produce a count of 2.475×10^{7} cfu.ml^{-1}. The

optical density at 600 nm from the neat bacterial suspension was taken as a reference value.

Dot blotting

The bacterial suspension was diluted 1:5, 1:10, 1:20 and 1:40 and used as antigen (Ag) for immunoblotting. The optical density (OD) of the Ag preparations was standardised between 0.14 to 0.23. Dots were blotted onto nitrocellulose membrane (pore diameter 0.45 μm) using bacterial cells as crude Ag preparations. Briefly, the nitrocellulose membrane was pre-wet in PBS (pH 7.4) for 10 minutes, then removed from the PBS solution and placed onto a 96 well Bio-Dot Microfiltration Apparatus (Bio-Rad). The membrane was allowed to cover all 96 holes and no bubbles were trapped in the membrane. The sample template of the apparatus was assembled on top of the membrane and properly sealed to avoid cross contamination between samples. An aliquot of 100 μl of PBS was applied to each well to re-hydrate the membrane, then removed by applying vacuum. A 100 μl inoculum of the Ag preparation was applied to each well of the membrane. Ag preparations were left overnight to pass through the membrane by gravity filtration at room temperature. After Ag preparations had completely drained, each well of the membrane was washed with 200 μl of PBS-T (0.05 % Tween 20). Membranes were removed from the apparatus and rinsed prior to blocking in 100 ml of a 5.0 % blocking solution (5.0 g of skimmed milk in 100 ml PBS-T) for 1 hr with slow shaking. Thereafter, the membrane was rinsed with 200 ml PBS three times and thereafter was ready to proceed with the application of antibodies as below. Negative controls (no Ag) were included in each assay.

Dot blotting onto nitrocellulose membrane (prepared as above) was used for titrating antibodies. Two fold serial dilutions from 1:10 to 1:320 of each antibody were made in PBS.

Appropriate concentration of antibodies diluted in PBS-T were placed into wells and incubated at room temperature with shaking for a minimum of 60 minutes, then rinsed with PBS-T three times.

Secondary Ab (Goat anti-Mouse conjugated with horseradish peroxidase-HRP) diluted 1:500 in PBS-T was then added and incubated as above. The membrane was rinsed in PBS-T three times, then given two final rinses with PBS. The reaction was developed using a freshly made substrate (Vector DAB kit).

The specificity of MAbs was tested on dot blots of the type strain ATCC 51139, field isolates of *B. pilosicoli,* and different species of the pig normal microflora such as *L. acidophilus*, *L. bulgaricus*, *Clostridium brevis*, *Escherichia coli*, total anaerobic bacteria, mixed enterococci, total aerobic bacteria and mixed coliform bacteria. Two-fold serial dilutions of each MAb were prepared in order to work out the titration for IMS. The avidity of each MAb was tested on crude preparations of bacterial cells of several field isolates with an OD between 0.150 to 0.240 at 600 nm. The preparations were blotted in duplicate onto nitrocellulose membrane as described above.

Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE)

A minigel system was used throughout the assay following the method of Hames and Rickwood, (1981). The separating gel was prepared at 10.0 % and the stacking gel at 4.0 %. The separating gel was prepared as follows: 4.85 ml distilled water; 2.5 ml 1.5M Tris-HCl pH 8.8; 100 µl 10.0 % (w/v) SDS; 2.5 ml 40 % acrylamide, and 5 µl (0.5 µl/ml) TEMED and 50 µl (5 µl/ml) 10.0 % ammonium persulphate (APS) were added at the very end for polymerisation, prior to loading into the 'sandwich' plates. After polymerisation of the separating gel, the stacking gel was prepared using 3.05 ml distilled water, 1.25 ml 1.5M Tris-HCl ph 8.8; 50 µl 10.0 % (w/v) SDS; 0.5 ml 40.0 % acrylamide, and 5 µl (0.5 µl/ml) TEMED and 25 µl (5 µl/ml) 10.0 % ammonium persulphate (APS), then poured into the 'sandwich' plates.

Preparation of bacterial cells for SDS-PAGE

Bacterial cells were washed in PBS as described previously, then the pellet was resuspended in PBS to give an OD between 0.6 and 0.8 (at 600 nm WL).

Bacterial suspensions were diluted 1:4 in sample buffer (50 µl of calibrated bacterial cells and 150 µl of sample buffer). The sample buffer (1 ml 0.5 M Tris-HCl, pH 6.8; 0.8 ml Glycerol; 1.6 ml 10.0 % (w/v) SDS; 0.4 ml 2-b-mercaptoethanol; 0.2 ml 0.05 % (w/v bromophenol blue), and 4.0 ml distilled water.
Bacterial suspensions were boiled for 5 min at 95 °C, put on ice immediately, and briefly centrifuged before loading onto the gel.

Electrophoresis conditions
The assembled gel cassette was placed in the gel tank and filled with running buffer (Appendix A). Samples (10 µl) were loaded alongside wide range protein marker (7.5 µl) (Rainbow coloured protein molecular weight markers, Amersham-Pharmacia, UK) as reference on each side of the gel. Separation of proteins was carried out at 200 V for 30 to 40 minutes. The gels were either stained or transferred onto nitrocellulose membrane for further analysis.

Staining of gels for proteins
Gels were stained for proteins using Brilliant Blue R staining solution (0.25 % w/v Brilliant Blue R, 40.0 % methanol, 7.0 % v/v acetic acid v/v final concentration). Gels were placed in the staining solution for 1.5 hrs, followed by de-staining by soaking the gels repeatedly in de-staining solution (40.0 % methanol, 7.0 % acetic acid) with gentle agitation. The de-staining solution was replaced as required until the desired intensity was achieved.

Electrophoretic transfer of proteins onto nitrocellulose membrane
Two sheets of blotting paper slightly bigger than the gel and a sheet of nitrocellulose membrane (0.45 µm) the size of the gel were soaked in transfer buffer [0.2 M Tris; 1.92 M Glycine; 20.0 % (v/v) methanol]. A 'sandwich' of the gel and nitrocellulose membrane was made between 2 sheets of blotting paper. The 'sandwich' was placed onto the cassette of the transfer apparatus, with the gels towards the cathode. The tank

of the transfer apparatus was filled with transfer buffer, and run at 60 Volts for 1.5 hrs and 0.52 Amp on ice to avoid heating.

Western blotting

The nitrocellulose membrane was removed from the 'sandwich' assembled in the transfer apparatus. Visualization of the Rainbow coloured protein marker on the nitrocellulose membrane was used to indicate successful transfer and to size the transferred proteins. Nitrocellulose membrane was rinsed with PBS-T for 2 times, then incubated in 5.0 % blocking buffer (5.0 % w/v skimmed milk in 100 ml PBS-T) overnight. After blocking, the nitrocellulose membrane was rinsed with PBS-T (0.1 %) once for 15 minutes, then twice for 5 minutes. The nitrocellulose membrane with proteins transferred was incubated with or without primary antibody. For detection, the nitrocellulose membrane was incubated with a 1:500 dilution of the secondary Ab (horseradish peroxidase conjugated) Goat anti-Mouse, Swine anti-Rabbit or Rabbit anti-Mouse for 1.5 hrs. After incubation with the secondary Ab, the nitrocellulose membrane was rinsed with PBS-T.

Development of reaction

Reactions were developed using two different methods.

1. Chemiluminescent substrate (SuperSignal, Pierce USA) method: 10 ml developer solution was prepared by mixing reagents as described by the manufacturer. The membranes were placed into the solution and left for 5 min, then removed to film foil to protect from light. Within 15 min after incubation with Super Signal, the membranes were exposed to emulsified photographic film (Kodak) for various exposure times.
2. Colourimetric method (DAB kit): developer solution (Vector) was made by mixing buffer, substrate and peroxidase according to manufacturer's instructions in a chosen volume i.e. 12 ml. The membrane was incubated until the reaction appeared (generally within 10 min), then rinsed with PBS and allowed to dry.

Immuno-magnetic separation technique

Bacterial cultures (bacterial suspension test)

Viable bacterial counts were calculated from the neat bacterials suspension in order to determine the level of detection (sensitivity) of the IMS assay. The two bacterial suspensions of *B. pilosicoli* ATCC 51139 contained 1.6 x 10^{10} and 3.3 x 10^{9} bacterial cells/ml, respectively. The bacterial suspension of *B. hyodysenteriae* isolate P944/14 contained 7.5 x 10^{6} viable bacterial cells/ml which were used to spike pig faeces for the IMS assays.

Faecal samples

Faeces from healthy pigs were diluted 1:20 (1 g of faeces in 20 ml PBS) and spiked with 30 µl of each dilution of the 10-fold diluted cultures of *B. pilosicoli* strain ATCC-51139 or *B. hyodysenteriae* isolate P944/14. The absence of *Brachyspira* spp. in the faeces used was confirmed by negative culture on BA plates.

Magnetic beads

Polystyrene magnetic beads covalently bound with rat MAb to mouse IgM (Dynabeads M-450 Rat anti-Mouse IgM, DYNAL, UK), or sheep MAb to rabbit IgG (Dynabeads M-280 Sheep anti-Rabbit IgG, DYNAL, UK) were used. The amount of magnetic beads used per reaction were 10^{7} of the M-450 or 1.5 x 10^{7} of the M-280 i.e. 25 µl of each as the original concentration of the products are ~4 x 10^{8} / ml or 6-7 x 10^{8} / ml, respectively. The magnetic beads were washed with washing buffer (Appendix A). Briefly, the vial was by vortexed, then the required amount of beads transferred into a washing tube (Eppendorf 1.5 ml). The tube was placed on a magnet for 2-3 minutes to capture the beads and the fluid pipetted off. The tube was removed from the magnet and the beads re-suspended in an excess volume of washing buffer. The washings were repeated 3 times, then the beads were re-suspended in coating buffer.

Coating magnetic beads

Dynabeads M-450 Rat anti-Mouse IgM precoated with a specific MAb that binds mouse IgM class. Dynabeads M-450 were coated either with the specific MAb to *B. pilosicoli* BJL/AC1 or the specific MAb to *B. hyodysenteriae* BJL/SH1. The coating procedure was as follows:

MAb to *B. pilosicoli* (BJL/AC1) or to *B. hyodysenteriae* (BJL/SH1) diluted 1:20 (20 µl specific Ab in 380 µl coating buffer) (Appendix A) were added to the washed beads Dynabeads M-450 (25 µl containing 10^7 magnetic beads approximately), vortexed, and incubated for 30 minutes, at room temperature with continuous slow rotation. The beads were collected using a magnet, and washed 4 times with washing buffer. Coated beads were resuspended in PBS in the original volume as extracted from the vial.

Coating rate

Antibody binding of the Rat anti-Mouse IgM to the specific MAb was verified by taking the OD of the supernatant before and after coating the Dynabeads M-450. If Ab binding occurred the reading on the OD should decrease whereas, if no binding took place the OD would remain unchanged after incubation.

IMS procedure

The direct and indirect methods of IMS were applied to separate the target bacterial cells ATCC-51139 type strain of *B. pilosicoli* and the isolate P944/14/00 of *B. hyodysenteriae* from the mixed bacterial cell suspension using the magnetic beads Dynabeads M-450 coated with MAb BJL/AC1 or MAb BJL/SH1, respectively. In the direct method (Appendix A), magnetic beads Dynabeads M-450 coated with the specific Ab were directly applied to the mixed suspension containing the diluted (1:20) pig faeces and the 10-fold diluted bacterial cell suspension of target cells (i.e. *B. pilosicoli* or *B. hyodysenteriae*). Incubation at room temperature for 30 minutes allowed the coated Dynabeads M-450 to bind the target cells. After incubation the Dynabeads M-450 were collected (i.e. the IMS product) using a magnet, washed with washing buffer 3 to 4

times, and re-suspended in 100 µl PBS. The direct IMS assay method was also performed using the Dynabeads M-280 coated with the PAb specific to *B. hyodysenteriae* as described above.

In the indirect method (Appendix A), the specific antibodies MAb BJL/AC1 or MAb BJL/SH1 were initially incubated for 1 hour (1st incubation) with the diluted pig faeces spiked with the 10-fold diluted series of bacterial cell suspension containing the target cells. This initial incubation allowed the specific antibody to bind to the target cells. Then, uncoated magnetic beads Dynabeads M-450 were added to the mixed cell suspension and incubated at room temperature for 30 minutes (2nd incubation). This secondary incubation allowed the Dynabeads M-450 to bind to the specific MAb, already bound to the target cells. After that incubation the Dynabeads M-450 were collected (i.e. the IMS product) using the magnet, then washed with washing buffer 3-4 times, and re-suspended in 100 µl PBS.

The direct IMS method is quicker than the indirect IMS method because the latter requires two incubations, however in general the indirect IMS method is considered to be more efficient for separating target cells from mixed cell suspensions.

The washed IMS product was used for plating out onto BA and TA (*Treponema* selective agar) plates.

Some assays were carried out without washing the magnetic beads after collection. Magnetic beads were collected, re-suspended in PBS and plated out directly on BA and TA plates.

Control samples (without IMS) comprised the diluted pig faeces spiked with the 10-fold diluted series of the bacterial suspension plated out onto BA and TA plates.

Recovery of target cells on culture media after IMS

The culture procedures were as follows:

10 µl inocula of the IMS product were plated out in triplicate onto BA and TA. BA was comprised of Columbia agar base No. 2 20 g, distilled water 455 ml plus 5 % sheep blood. TA was made with a selective supplement comprising spectinomycin

dehydrochloride 4.0 g, vancomycin hydrochloride (500,000 IU) 0.125 g, colistin methane sulphonate 13100 IU/mg 0.125 g and distilled water 100 ml. A control plate was inoculated with 10 µl of the original bacterial suspension for monitoring the viability of the culture on each assay. Inocula from the supernatant of each of the washings of the IMS product were also plated out in order to determine whether the target cells (*B. pilosicoli* and *B. hyodysenteriae*) were washed out in each washing. Inoculated plates were incubated anaerobically at two different temperatures (37 °C and 42 °C) for 3-4 days. Smear preparations were made from cultures obtained following the IMS and from control plates (without IMS) to confirm the purity of cultures. Smear preparations were Gram stained and observed by microscopy.

PCR testing from IMS product and control samples no IMS (spiked faeces)

DNA extraction

From both IMS product and control (without IMS) samples an aliquot of 50 µl of each was used to extract the DNA followed by PCR to determine the sensitivity of both methods for detecting the target cells in spiked faeces. The aliquots were taken from the same sample of which the inocula for cultures was taken from. Aliquots were boiled for 5 min to allow separation of magnetic beads (in the case of IMS samples) from biological material. Samples were centrifuged at 5000 rpm for 5 min and the supernatant discarded, then the pellet was resuspended in 50 µl of distilled water. Genomic DNA was extracted using Qiagen columns (Qiagenamp Mini kit, Qiagen Ltd, UK). DNA extraction was carried out according to the tissue protocol of the QIAamp DNA mini kit (Appendix A).

The presence of DNA after extraction from samples was confirmed by running 10 µl of the extracted DNA on a 1.0 % agarose gel (Appendix A).

PCR for detection of Brachyspira sp.

The sensitivity of the IMS technique as compared with control samples (without IMS) was evaluated by a PCR test specific for the porcine intestinal spirochaetes of the genus *Brachyspira*. The sensitivity of the IMS as compared with no IMS was determined by the amplification of the DNA from both sample types containing known numbers of *B. pilosicoli* and *B. hyodysenteriae* cells. The PCR amplifies the DNA of *Brachyspira sp.* by using a combination of two primers which generate a characteristic DNA fragment of 555 bp in size (Teran-Dianderas, 1997). The PCR test followed by enzymatic digestion of the amplified fragment was used to differentiate the species *B. hydysenteriae*, *B. innocens* and *B. pilosicoli*. This method of identification has been used by the Scottish Agricultural College Veterinary Science Division, Edinburgh as a routine diagnostic test for porcine intestinal spirochaetes since 1996.

Primers and PCR conditions

The primers SF1 (24 mer) and SR1 (20 mer) were used for the PCR test, these primers amplify a highly conserved region found between the nucleotides 874 and 1429 of the 23S rRNA gene of *Brachyspira sp.* The sequences of the primers are SF1 5' CAGCTAAGGTCCCAAAATCTATGT 3' and SR1 5' GAACCCGAAAGCCCAGTCAC 3' which generate a fragment of 555 bp in size (Teran-Dianderas, 1997). The PCR reaction consisted of a master mix (MegaMix-Blue Microzone Ltd, UK) containing 200 µl of each dNTPs, 2.5 mM $MgCl_2$, *Taq* polymerase (1 U / 50 µl), blue dye and stabiliser, in 30.5 µl final volume per reaction. Each reaction had MegaMix-Blue (25.5 µl), primer SF1 (1.5 µl; 0.2 mM), primer SR1 (1.5 µl; 0.2 mM) and DNA template (2 µl).

Cycling was carried out for one round of 4 minutes denaturation (94 °C), 1 min annealing (60 °C) and 2 min extension (72 °C), followed by 39 cycles of 1 minute denaturation, 1 minute annealing and 2 minutes extension at the same temperatures. After the thermocycling was completed samples were preserved at 4 °C.

Displaying and visualisation of PCR products

PCR products were electrophoresed on 1.5 % agarose gel (Appendix A) which consisted of 1.5 g agarose in 100 ml 1X TAE buffer (Appendix A) pH 8.0.

Enzymatic digestion of Brachyspira species PCR products

Although the isolates used in IMS assays were identified as *B. pilosicoli* and *B. hyodysenteriae*, and the pig faeces came from healthy pigs, the enzymatic digestion was done to confirm that no other *Brachyspira* species was contained in the samples.

The enzymatic digestion was carried out following the method by Teran-Dianderas (1997), which consisted in digestion of PCR products using the restriction enzyme *Hph* I (New England Biolabs, UK) with a recognition site 5' GCTGA 3'. The reaction components included the buffer supplied with enzyme by the manufacturer that contains 50 mM potassium acetate, 29 mM Tris-acetate, 10mM magnesium acetate, 1 mM dithiothreitol (pH 7.9). The reaction was carried out in 0.5 ml microtubes with a final volume of 20.5 µl of which enzyme buffer 2 µl, enzyme *Hph* I 0.5 µl, PCR product 3 µl and filtered water 15 µl. If the band of the PCR product was weak the amount of PCR product was increased to 10 µl. After adding the reagents tubes were incubated at 37 °C for 75 minutes.

The enzymatic digestion of PCR products produces characteristic fragments from each of the *Brachyspira* species, *B. hyodysenteriae* three fragments 298, 201 and 51 bp; *B. innocens* two fragments 290 and 267 bp, and *B. pilosicoli* three fragments 472, 57 and 29 bp. Digestion products were electrophoresed on 10 % polyacrylamide gels, and silver stained (Appendix A).

Results

Titration of MAbs

MAbs were titrated using dot blotting with Ag preparations of *B. pilosicoli* and *B. hyodysenteriae*. Bacterial suspensions were prepared by harvesting the growth of two BA plates of which the OD at 600 nm was taken to standardise the concentration of Ag preparations. The OD values of 13 and 14 bacterial suspension preparations of *B. pilosicoli* and *B. hyodysenteriae*, respectively were not significantly different (Appendix B).

The cut off point titre (i.e. the highest dilution at which MAb reactivity to Ag was detected) of MAbs BJL/AC1 and BJl/AC1 specific to *B. pilosicoli* and to *B. hyodysenteriae*, respectively was 1:160 for both MAbs (Fig. 3.1).
Since the 1:20 dilution gave better intensity of reaction compared to the 1:40 dilution, this dilution was chosen for coating the magnetic beads for IMS assays.

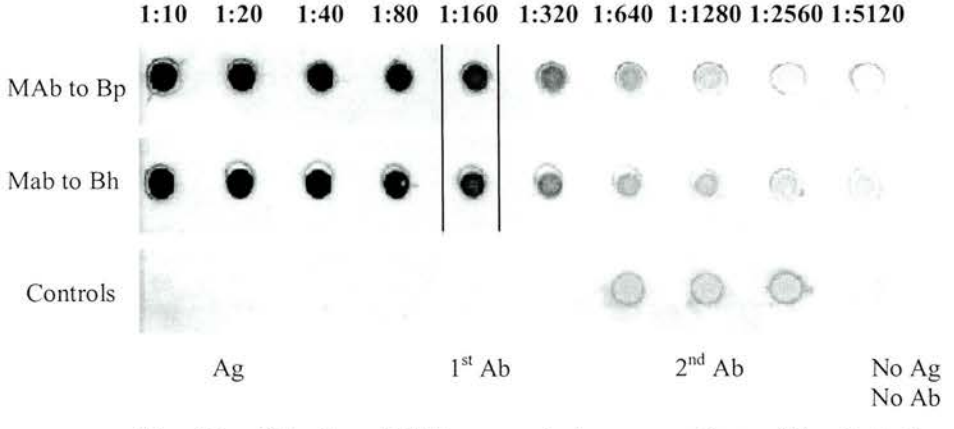

Fig. 3.1. Titration of MAbs on crude Ag preparations of *B. pilosicoli* and *B. hyodysenteriae*. The cut off point is marked between the parallel bars.

Specificity of MAbs

MAb BJL/AC1 specific to *B. pilosicoli* showed different levels of reaction intensity with various field isolates of *B. pilosicoli* at the dilution tested (1:20). For instance, a weaker reaction of the MAb BJL/AC1 was observed with the reference strain ATCC 51139 compared to a stronger reaction observed with some of the isolates of *B. pilosicoli* (Fig. 3.2). Although MAb BJ1/AC1 was raised against *B. pilosicoli*, it still showed some reactivity to isolates of the normal pig gut flora (data not shown), which may affect its usefulness in IMS.

The MAb BJL/SH1 to *B. hyodysenteriae* showed a more defined positive reaction as no difference in the level of reaction intensity was observed between the two isolates of *B. hyodysenteriae* tested, and no cross-reaction was observed with any of the isolates of *B. innocens* or *B. pilosicoli* (Fig. 3.3). Of the organisms from the normal pig gut flora, a very weak reaction was observed to some of the isolates of the normal pig flora such as *Cl. brevis* and to TAN but no reaction was observed to *E. coli* isolates using this MAb (data not shown).

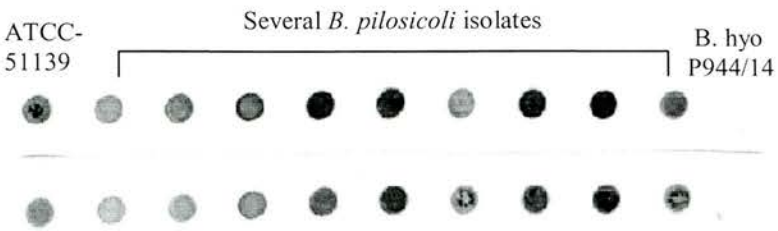

Fig. 3.2. Specificity of MAb to *B. pilosicoli*, showing positive reaction with the reference strain ATCC-51139 and several isolates of *B. pilosicoli*. No reaction is seen with the isolate P944/14/00 of *B. hyodysenteriae*.

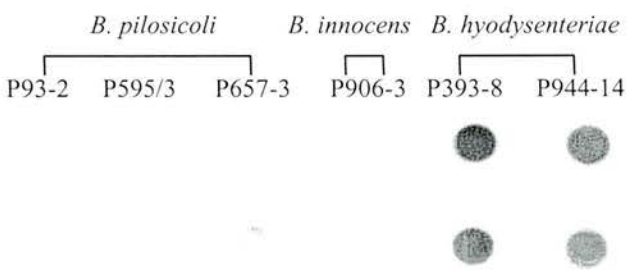

Fig. 3.3. Specificity of MAb to *B. hyodysenteriae*, showing positive reaction with the isolates P393/8 and P944/12 of *B. hyodysenteriae*. No reaction is seen with isolates of *B. pilosicoli* or *B. innocens*

Specificity of the PAb

The PAb raised against *B. hyodysenteriae* diluted 1:20 showed reactivity to *B. hyodysenteriae* and also to some isolates of *B. pilosicoli*, the reaction was stronger with the dog isolate K19, P646/1 and P676/1 (Fig 3.4). The PAb did not bind the reference strain ATCC 51139 of *B. pilosicoli* nor the isolates of *B. innocens*. These results were consistent on three replicate tests.

While performing the assay to determine the specificity of the PAb a positive reaction was observed with chemi-luminecense or DAB kit on control reactions for *B. pilosicoli* [i.e. Ag incubated with Goat anti-Rabbit peroxidase conjugated (Dako, Denmark)], but that positive reaction was not observed with controls for either *B. innocens* or *B. hyodysenteriae*, suggesting that goat immunoglobulins might recognise protein antigens of *B. pilosicoli* (Fig. 3.4).

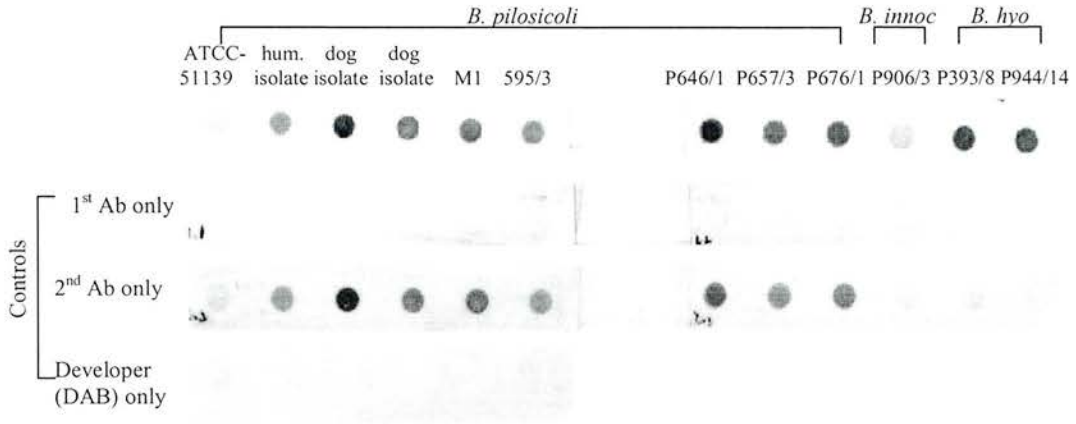

Fig. 3.4 Polyclonal Abs species specific to *B. hyodysenteriae* showing positive reaction with isolates P393/8 and P944/14 of *B. hyodysenteriae* but also with the type strain ATCC-51139 and isolates of *B. pilosicoli*, and one isolate of *B. innocens*. On controls, the 2nd Ab (goat anti-rabbit) shows reation with Ag preparations of *B. pilosicoli* isolates but not with those of *B. innocens* or *B. hyodysenteriae* isolates.

Further testing using conjugated Swine anti-Rabbit (Dako, Denmark) as the second Ab produced a weak positive reaction, whereas no positive reaction was observed in assays with the secondary Ab Rabbit anti-Mouse. Further dot blots and Western blotting assays were carried out using isolates of the three species of *Brachyspira* with inclusion of further controls to ensure that the positive reaction observed with Goat anti-Mouse was not a false positive. Controls included second Ab only [Goat anti-Rabbit, Rabbit anti-Mouse or Swine anti-Rabbit (Dako, Denmark)], neither first Ab nor second Ab, and first Ab only.

Western blotting

Conjugated second Ab Goat anti-Mouse reacted positively and clearly with an Ag of molecular size approximately 50 KDa from the two dog isolates K19 and K22, and with the isolate P32/2/97 of *B. pilosicoli* (Fig 3.5). On repeat assays using a different batch of secondary Ab a similar positive reaction to 50 kDa Ag protein was observed with the isolates indicated above and also with other isolates of *B. pilosicoli* (P93/2/94, P595/3/00, P657/8/00 and P676/2/00) (Fig 3.6). Using the conjugated Goat anti-Rabbit reactivity towards the 50 KDa Ag remained positive. The positive reaction was observed after three assays, however, no reaction towards the Ag proteins of *B. innocens* or *B. hyodysenteriae* was detected. Similar positive results were found in assays performed with the second Ab Swine anti-Rabbit peroxidase conjugated but the reaction was weaker, indicating that swine immunoglobulins were also recognising a protein of similar molecular size (50 KDa) in *B. pilosicoli* isolates. No reaction with that second Ab Swine anti-Rabbit was observed towards Ag proteins of neither *B. innocens* nor *B. hyodysenteriae* isolates. Although this finding suggested that *B. pilosicoli* may express an immunoglobulin binding protein, this was not further pursued as it was not the aim of the current study.

The second Ab Rabbit anti-Mouse peroxidase conjugated did not show reactivity towards any protein Ag of *B. pilosicoli*, *B. innocens* nor *B. hyodysenteriae*.

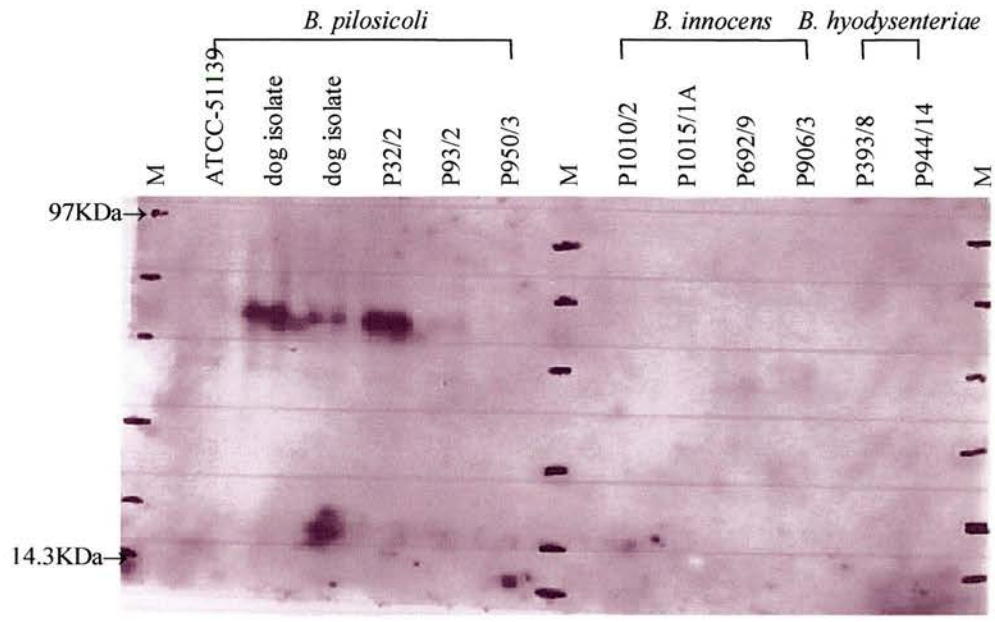

Fig. 3.5 Labelled goat immonoglobulines (used as 2nd Ab in Western blots) recognising a protein Ag of 50 KDa approximately on three isolates of *B. pilosicoli*. No reaction is seen with isolates of *B. innocens* or *B. hyodysenteriae*.

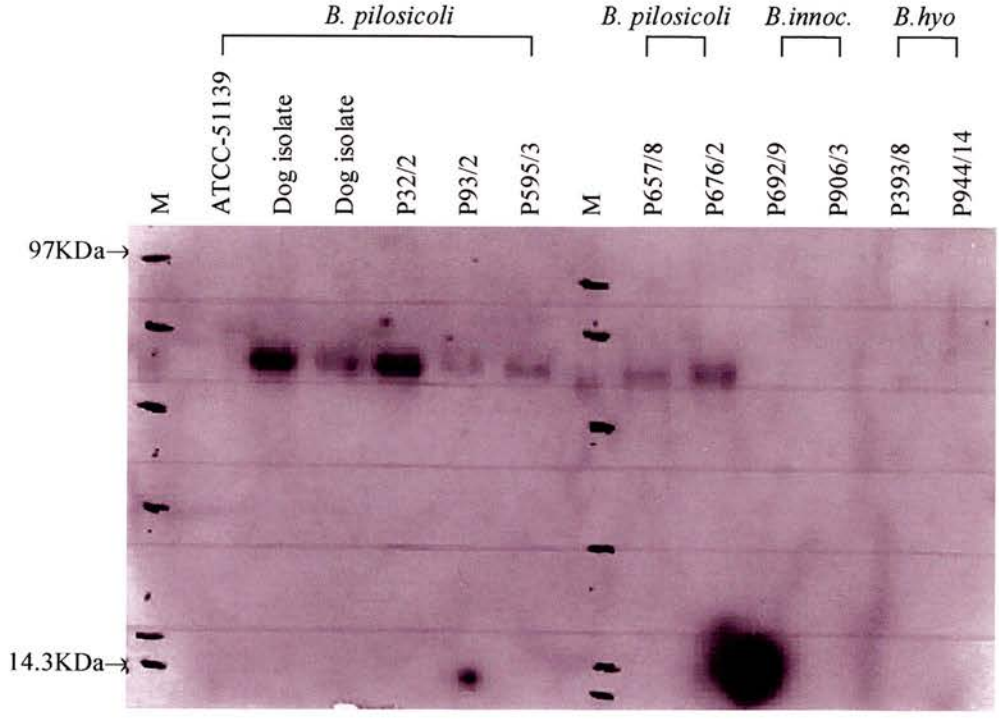

Fig. 3.6 Labelled goat immunoglobulins (used as 2nd Ab in Western blotting) recognising a protein Ag of 50 KDa on several isolates of *B. pilosicoli*. No reaction is seen with isolates of *B. innocens* or *B. hyodysenteriae*

Coating rate

To confirm efficient coating of magnetic beads Dynadeads M-450, the coating rate of magnetic beads used in IMS (direct method) assays was monitored as shown in Table 3.1. A decrease in the OD reading was observed on the supernatant of collected beads after being coated with the desired specific Ab, indicating that Ab binding to the mouse IgM class by the specific Rat anti-Mouse conjugated to Dynabeads M-450 had occurred. A dilution of 1:20 of MAb BJL/AC1 and BJL/SH1 showed effective binding to magnetic beads. Therefore this dilution was used to assess the IMS.

Table 3.1. OD values of coating rate of Dynabeads M-450.

Ab/dilution	OD 280 nm	
	Before coating	after coating
MAb BJL/AC1 1:20	0.360	0.284
MAb BJL/SH1 1:20	0.216	0.206

Efficacy of culture and PCR methods for detection of *B. pilosicoli* ATCC-51139 and *B. hyodysenteriae* isolate P944/14/00 with IMS and control (without IMS) on BA and TA plates (selective culture medium).

Cultures on BA and TA plates inoculated with IMS products (direct and indirect methods) and controls (without IMS) inocula from spiked pig faeces with *B. pilosicoli* ATCC-51139, and *B. hyodysenteriae* isolate P944/14 were compared. The growth characteristics and the sensitivity of isolating *B. pilosicoli* and *B. hyodysenteriae* on plates incubated at 42 °C were superior than those incubated at 37 °C (Fig. 3.7). Therefore, all the BA and TA plates of IMS assays were incubated at 42 °C.

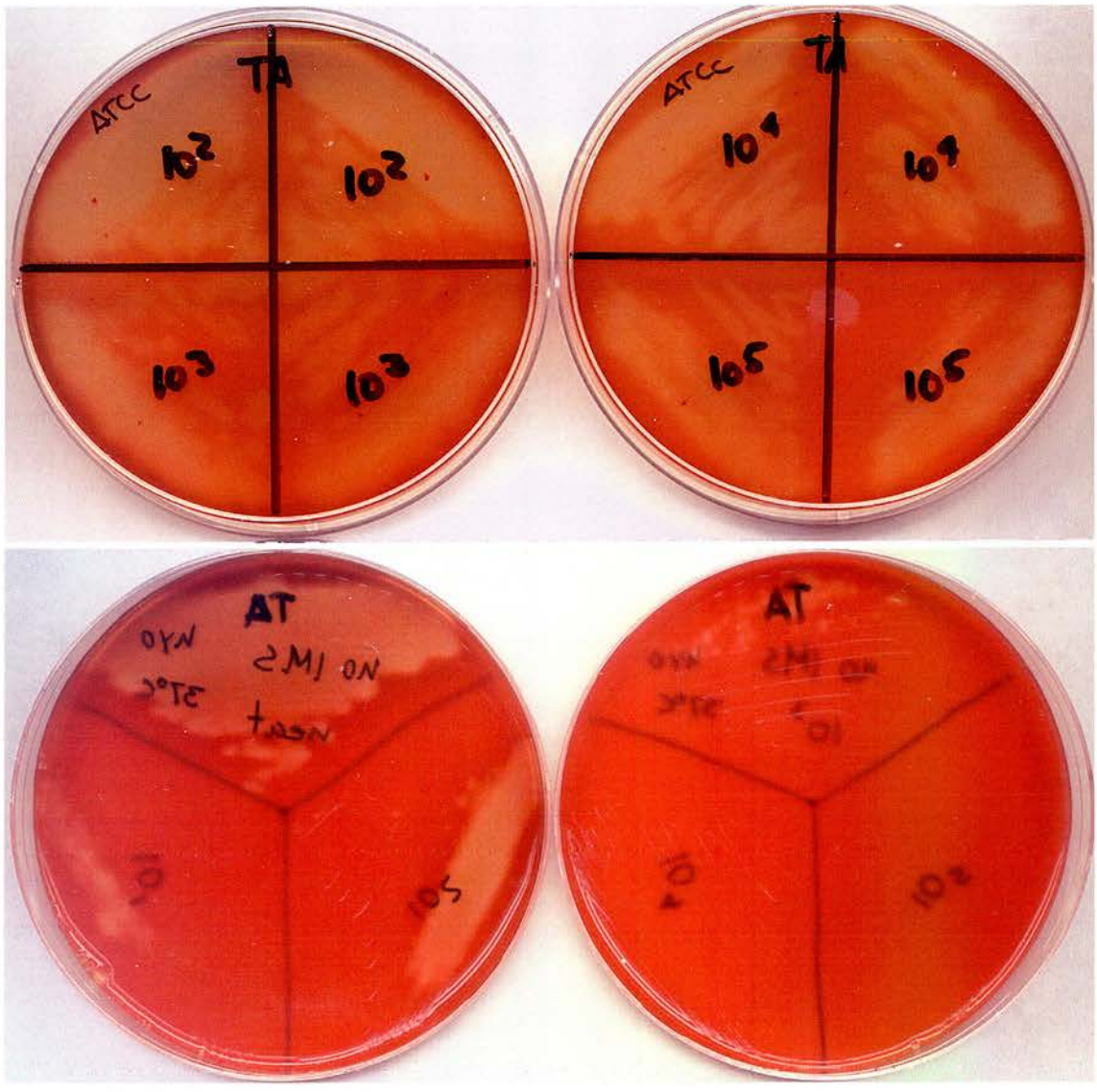

Fig.3.7. Top, growth of *Brachyspira pilosicoli* on TA plates (incubated at 42 °C), inocula (from dilutions 10^2 to 10^5 two replicates each) taken from a bacterial suspension containing 1.6 x 10^{10} viable cells of *B. pilosicoli* per ml; bottom, growth of *B.hyodysenteriae* on TA plates (incubated at 37 °C) , inocula (from the neat to dilution 10^5) taken from a bacterial suspension containing 7.5 x 10^6 viable cells of *B. hyodysenteriae* per ml.

IMS direct method for B. pilosicoli on BA plates (Fig. 3.8 and Table 3.2).

The IMS product inocula of every dilution of the IMS assay using *B. pilosicoli* ATCC-51139 and Dynabeads M-450 coated with MAb BJL/AC1 produced a mixed growth culture on BA plates, and no characteristic growth of *B. pilosicoli* was observed on these plates. Conversely, the control for that assay the without IMS inocula had a relative sensitivity for recovering *B. pilosicoli* on BA plates at the level of dilution 10^2, corresponding to 4.8×10^6 total count of viable bacterial cells, although that growth on BA plates was mixed with bacteria of the pig normal microflora as confirmed microscopically on smear preparations. The control (without IMS) at and higher dilutions than 4.8×10^5 inocula produced mixed growth with no detectable characteristic growth of *B. pilosicoli* (Table 3.2. and Fig. 3.8).

Fig. 3.8. Growth of *B. pilosicoli* from inocula of IMS direct and indirect methods on BA plates (incubated at 42°C), showing mixed culture. Left, Inocula (3 replicates)of the IMS direct method taken from a bacterial suspension containing 1.6×10^{10} of viable cells of *B. pilosicoli* per ml. Right, inocula (3 replicates)of the IMS indirect methodtaken from a bacterial suspension containing 5 x 10^9viable cells of *B. pilosicoli* per ml.

Table 3.2. IMS direct method for detection of *B. pilosicoli* in spiked pig faeces.

Dilution	Total count of viable bacterial cells	Culture medium				PCR results	
		BA		TA		IMS	*no IMS*
		IMS	*No IMS*	IMS	*no IMS*		
Neat	4.8×10^8	Mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)	ND	ND
$*10^1$	4.8×10^7	Mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)	+	+
$*10^2$	4.8×10^6	Mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)	+	+
$*10^3$	4.8×10^5	Mixed	Mixed	Growth no mixed (1/3)	Growth no mixed (1/3)	+	+
$*10^4$	4.8×10^4	Mixed	Mixed	NG	Growth no mixed (3/3)	-	+
$*10^5$	4.8×10^3	Mixed	Mixed	NG	Growth no mixed (3/3)	-	-
$*10^6$	4.8×10^2	Mixed	Mixed	NG	Growth no mixed (2/3)	-	-
$*10^7$	4.8×10^1	Mixed	Mixed	NG	NG	-	-

*10-fold dilutions were made from a bacterial suspension containing 1.6×10^{10} per ml of viable cells of *B. pilosicoli*; Growth but mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* mixed with normal intestinal bacteria; Growth no mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* only; Mixed: mixed culture containing porcine normal intestinal bacteria; NG: no growth observed on plates; ND: not done; + or – PCR result.

IMS indirect method for B. pilosicoli on BA plates

The inocula of the IMS product using the MAb JBL/AC1 and Dynabeads M-450 as described above was three 10-fold dilutions more sensitive than the IMS direct method for recovery of *B. pilosicoli* on BA plates. Growth developed at the level of dilution 10^2 corresponding to 1.5×10^6 total count of viable bacterial cells of *B. pilosicoli* ATCC-51139, although, again that growth of *B. pilosicoli* on BA plates was mixed with bacteria of the pig normal microflora. The control (without IMS) for that assay had higher relative sensitivity than both the IMS (indirect method) and the IMS (direct method) on BA plates. Characteristic growth of *B. pilosicoli* developed at the level of dilution 10^4,

corresponding to 1.5 x 10^4 total count of viable bacterial cells, although, the growth of *B. pilosicoli* ATCC-51139 on BA plates was also mixed with bacteria of the pig normal flora (Table 3.3. and Fig. 3.8).

Table 3.3. IMS indirect method for detection of *B. pilosicoli* in spiked pig faeces.

Dilution	Total count of viable bacterial cells	Culture medium				PCR results
		BA		TA		
		Indirect IMS	*No IMS*	Indirect IMS	*No IMS*	Indirect IMS
Neat	1.5 x 10^8	Growth but mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)	ND
*10^1	1.5 x 10^7	Growth but mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)	+
*10^2	1.5 x 10^6	Growth but mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)	+
*10^3	1.5 x 10^5	Mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)	+
*10^4	1.5 x 10^4	Mixed	Growth but mixed	Growth no mixed (2/3)	Growth no mixed (3/3)	-
*10^5	1.5 x 10^3	Mixed	Mixed	Growth no mixed (13/3)	Growth no mixed (3/3)	-
*10^6	1.5 x 10^2	Mixed	Mixed	NG	Growth no mixed (3/3)	-
*10^7	1.5 x 10^1	Mixed	Mixed	NG	NG	ND

*10-fold dilutions were made from a bacterial suspension containing 5 x 10^9 per ml of viable cells of *B. pilosicoli*; Growth but mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* mixed with normal intestinal bacteria; Growth no mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* only; Mixed: mixed culture containing porcine normal intestinal bacteria; NG: no growth observed on plates; ND: not done; + or – PCR result.

IMS direct method for B. hyodysenteriae on BA plates

Both the IMS product and the control (without IMS) inocula of the IMS assay (direct method) using *B. hyodysenteriae* isolate P944/14 and Dynabeads M-450 coated with the MAb BJL/SH1 produced mixed culture on BA plates. No characteristic growth of *B. hyodysenteriae* was observed on those plates (Table 3.4. and Fig. 3.9).

Table 3.4. IMS direct method for detection of *B. hyodysenteriae* in spiked pig faeces.

Dilution	Total count	Culture medium				PCR results	
		BA		TA			
		IMS	*no IMS*	IMS	*no IMS*	IMS	*no IMS*
Neat	2.5×10^5	Mixed	Mixed	Growth no mixed (3/3)	Growth no mixed (3/3)	ND	ND
$*10^1$	2.5×10^4	Mixed	Mixed	Growth no mixed (2/3)	Growth no mixed (3/3)	+	+
$*10^2$	2.5×10^3	Mixed	Mixed	NG	Growth no mixed (3/3)	+	+
$*10^3$	2.5×10^2	Mixed	Mixed	NG	Growth no mixed (3/3)	-	+
$*10^4$	2.5×10^1	Mixed	Mixed	NG	Growth no mixed (1/3)	-	+?
$*10^5$	2.5	Mixed	Mixed	NG	NG	-	-
$*10^6$		Mixed	Mixed	NG	NG	-	-
$*10^7$		Mixed	Mixed	NG	NG	ND	ND

*10-fold dilutions were made from a bacterial suspension containing 7.5×10^6 per ml of viable cells of *B. hyodysenteriae*; Growth but mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* mixed with normal intestinal bacteria; Growth no mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* only; Mixed: mixed culture containing porcine normal intestinal bacteria; NG: no growth observed on plates; ND: not done; + or – PCR result; +? faint band of PCR product displayed on gel..

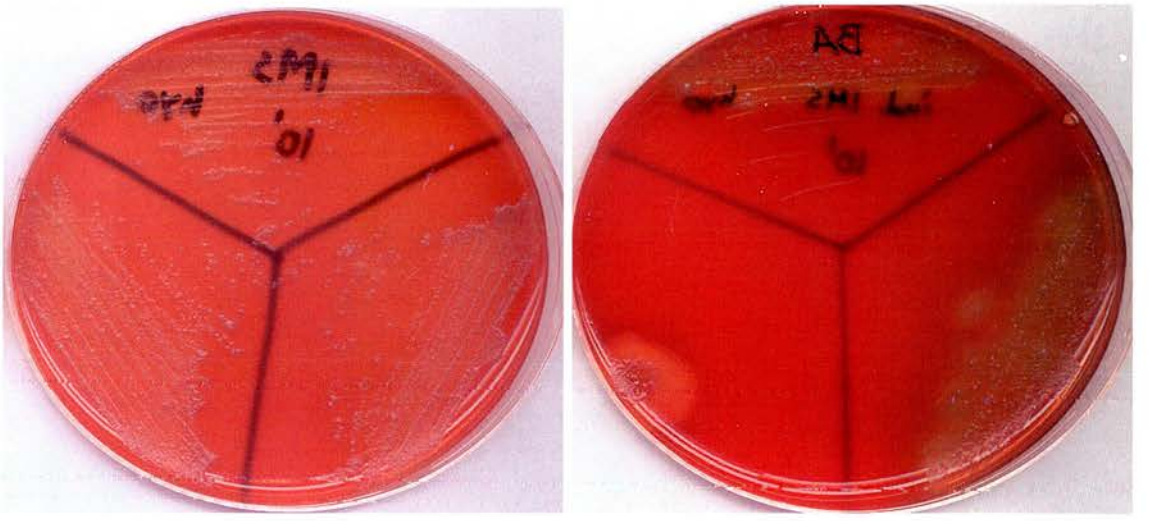

Fig. 3.9. Growth of *B. hyodysenteriae* from inocula of IMS direct method (right plate) and indirect method (left plate) on BA (incubated at 42 C), showing mixed culture. Left, inocula (3 replicates) from a bacterial suspension containing 7.5×10^6 viable cells of *B. hyodysenteriae*. Right, inocula (3 replicates) from a bacterial suspension containing 3.3×10^6 viable cells of *B. hyodysenteriae*.

IMS indirect method for B. hyodysenteriae on BA plates

Both the IMS product and the control (without IMS) inocula of the IMS assay (indirect method) using *B. hyosysenteriae* isolate P944/14, the MAb BJL/SH1 and Dynabeads M-450 had the same relative sensitivity (at dilution 10^4) for recovering *B. hyodysenteriae* on BA plates. This corresponded to 9.9 x 10^1 total count of viable bacterial cells of *B. hyodysenteriae* isolate P944/14 which developed the characteristic growth. Again, the growth of *B. hyodysenteriae* on BA plates was mixed with bacteria of the pig normal flora (Table 3.5 and Fig. 3.9).

Table 3.5. IMS indirect method for detection of *B. hyodysenteriae* in spiked pig faeces.

Dilution	Total count of viable bacterial cells	Culture medium			
		BA		TA	
		Indirect IMS	*no IMS*	Indirect IMS	*no IMS*
Neat	9.9 x 10^5	Growth but mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)
*10^1	9.9 x 10^4	Growth but mixed	Growth but mixed	Growth no mixed (3/3)	Growth no mixed (3/3)
*10^2	9.9 x 10^3	Growth but mixed	Growth but mixed	Growth no mixed (2/3)	Growth no mixed (3/3)
*10^3	9.9 x 10^2	Growth but mixed	Growth but mixed	NG (0/3)	Growth no mixed (3/3)
*10^4	9.9 x 10^1	Growth but mixed	Growth but mixed	NG (0/3)	Growth no mixed (3/3)
*10^5	9.9	Mixed	mixed	NG (0/3)	Growth no mixed (3/3)
*10^6		Mixed	mixed	NG (0/3)	NG (0/3)
*10^7		Mixed	mixed	NG (0/3)	NG (0/3)

*10-fold dilutions were made from a bacterial suspension containing 3.3 x 10^7 per ml of viable cells of *B. hyodysenteriae*; Growth but mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* mixed with normal intestinal bacteria; Growth no mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* only; Mixed: mixed culture containing porcine normal intestinal bacteria; NG: no growth observed on plates.

IMS direct method for B. pilosicoli on TA plates

The growth on all TA plates was pure culture as confirmed by microscopic examination of smear preparations of plate samples. Direct IMS using *B. pilosicoli* ATCC-51139 and Dynabeads M-450 coated with the MAb BJL/AC1 had a sensitivity for recovering *B. pilosicoli* on TA plates at the level of dilution 10^3 corresponding to 4.8 x 10^5 total count of viable bacterial cells of *B. pilosicoli*. That relative sensitivity was two 10-fold dilutions higher than the IMS product inoculated on BA plates and the growth on TA was pure culture as confirmed on smears. However, dilutions at or higher than 10^3 of the IMS inocula did not produce further growth. The control (without IMS) had even a higher relative sensitivity for recovering *B. pilosicoli* on TA plates since growth developed at the level of dilution 10^6 corresponding to 4.8 x 10^2 total viable count of bacterial cells of *B. pilosicoli* which was three 10-fold higher dilutions than the IMS product (Table 3.2. and Fig. 3.10).

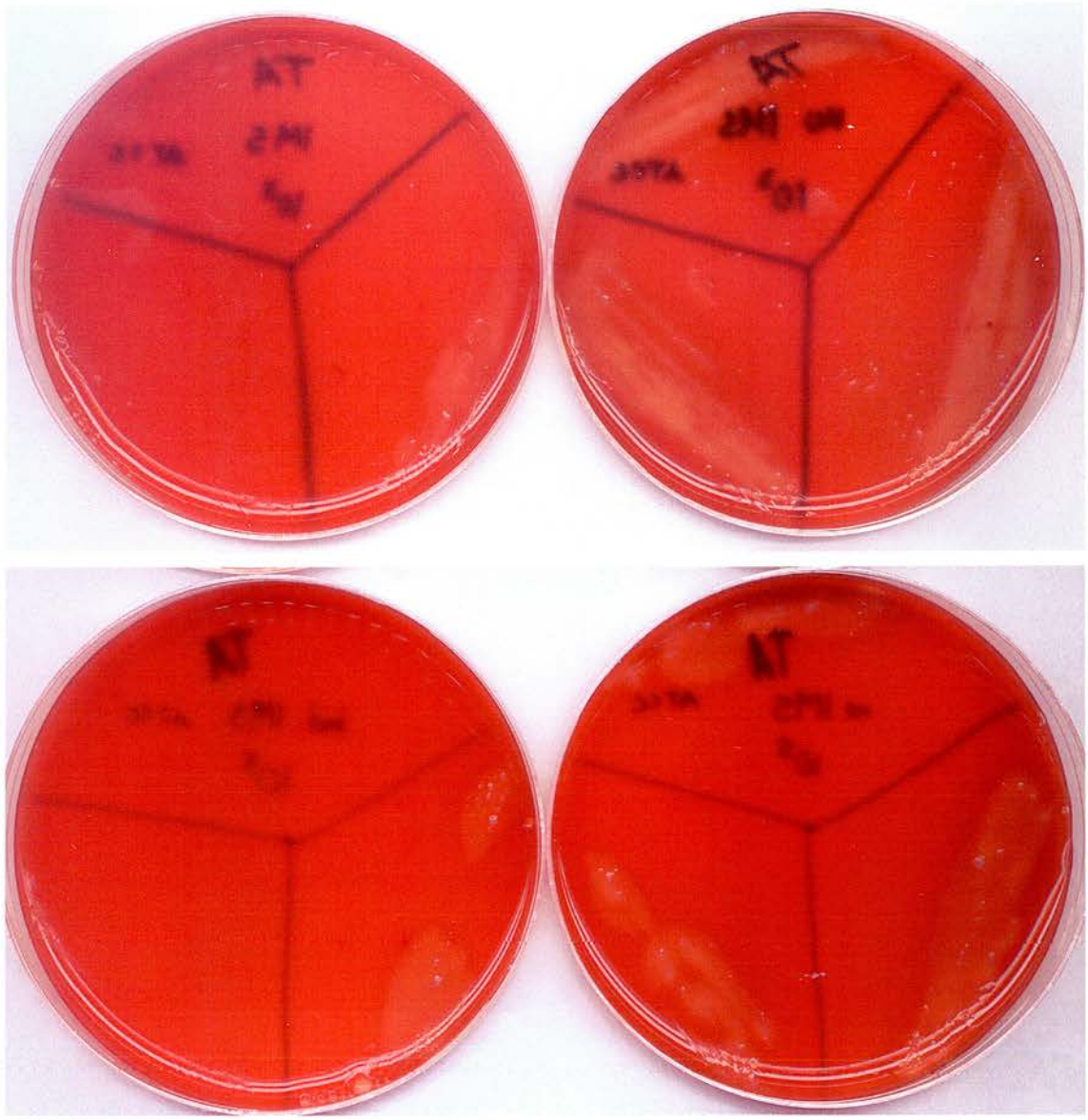

Fig. 3.10. Growth of *B. pilosicoli* from inocula of IMS direct method (top left), indirect method (bottom left), and control without IMS (right top and bottom) on TA plates (incubated at 42 C), showing pure culture. Top, inocula (3 replicates) taken from dilution 10^3 of a bacterial suspension containing 1.6 x 10^{10} per ml viable cells of *B. pilosicoli*. Bottom, inocula (3 replicates) taken from dilution 10^5 of a bacterial suspension containing 5.6 10^9 per ml viable cells of *B. pilosicoli*.

Repeated IMS assays revealed that the relative sensitivity of the IMS assays to recover the target cells (*B. pilosicoli* or *B. hyodysenteriae*) was not markedly higher than the control (without IMS) inocula. This could have been because some of the target cells were washed off in each of the washings (3-4 washings as recommended in the manufacturer's protocol) carried out on the IMS product. Therefore, an IMS (direct method for *B. pilosicoli*) assay using *B. pilosicoli* ATCC-51139 and Dynabeads M-450 coated with MAb BJL/AC1 without washing the IMS product was run in parallel with a normal assay procedure. The inocula of the washings were also plated out onto TA plates to determine whether *Brachyspira* cells were contained in those washings. The results of that assay showed two 10-fold dilution higher sensitivity for recovering *B. pilosicoli* on TA plates as compared to the normal procedure of washing the IMS product. The characteristic growth of *B. pilosicoli* developed at the level of dilution 10^5 corresponding to 9.9×10^1 total count of viable bacterial cells of *B. pilosicoli* ATCC-51139 (Table 3.6). Moreover, *B. pilosicoli* ATCC-51139 was also recovered from each of the three of the washings showing different degrees of sensitivity for recovering *B. pilosicoli* on TA plates, indicating that a number of the target cells were indeed washed off in each of the washings. The relative sensitivities of recovering *B. pilosicoli* ATCC-51139 on TA plates from the first, second and third washings were at the level of dilutions 10^5, 10^5 and 10^3 corresponding to 9.9×10^1, 9.9×10^1 and 9.9×10^3 total count of viable bacterial cells of *B. pilosicoli* ATCC-51139, respectively (Table 3.6 and Fig. 3.11). However, the relative sensitivity of the control (without IMS) inocula was still higher than the IMS product (no washing) and the first washing since growth of *B. pilosicoli* ATCC-51139 was developed at the level of dilution 10^5 corresponding to 9.9 viable cells of *B. pilosicoli* ATCC51-139 (Table 3.5).

Table 3.6. IMS (direct method) for *B.pilosicoli* without washing and its recovery on TA plates from the washings.

Dilution	Total count of viable bacterial cells	Culture medium					PCR results
		TA					
		IMS	IMS no washing	*Washings*			
				1	2	3	IMS no washing
Neat	9.9×10^6	Growth no mixed (3/3)	Growth no mixed (3/3)	Growth no mixed	Growth no mixed	Growth no mixed	ND
$*10^1$	9.9×10^5	Growth no mixed (3/3)	Growth no mixed (3/3)	Growth no mixed	Growth no mixed	Growth no mixed	+
$*10^2$	9.9×10^4	Growth no mixed (3/3)	Growth no mixed (3/3)	Growth no mixed	Growth no mixed	Growth no mixed	+
$*10^3$	9.9×10^3	Growth no mixed (3/3)	Growth no mixed (3/3)	Growth no mixed	Growth no mixed	Growth no mixed	+
$*10^4$	9.9×10^2	NG	Growth no mixed (3/3)	Growth no mixed	Growth no mixed	NG	-
$*10^5$	9.9×10^1	NG	Growth no mixed (3/3)	Growth no mixed	Growth no mixed	NG	-
$*10^6$	9.9	NG	NG	NG	NG	NG	-
$*10^7$		NG	NG	NG	NG	NG	ND

*10-fold dilutions were made from a bacterial suspension containing 3.3×10^8 per ml of viable cells of *B. pilosicoli*; Growth but mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* mixed with normal intestinal bacteria; Growth no mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* only; Mixed: mixed culture containing porcine normal intestinal bacteria; NG: no growth observed on plates; ND: not done; + or – PCR result.

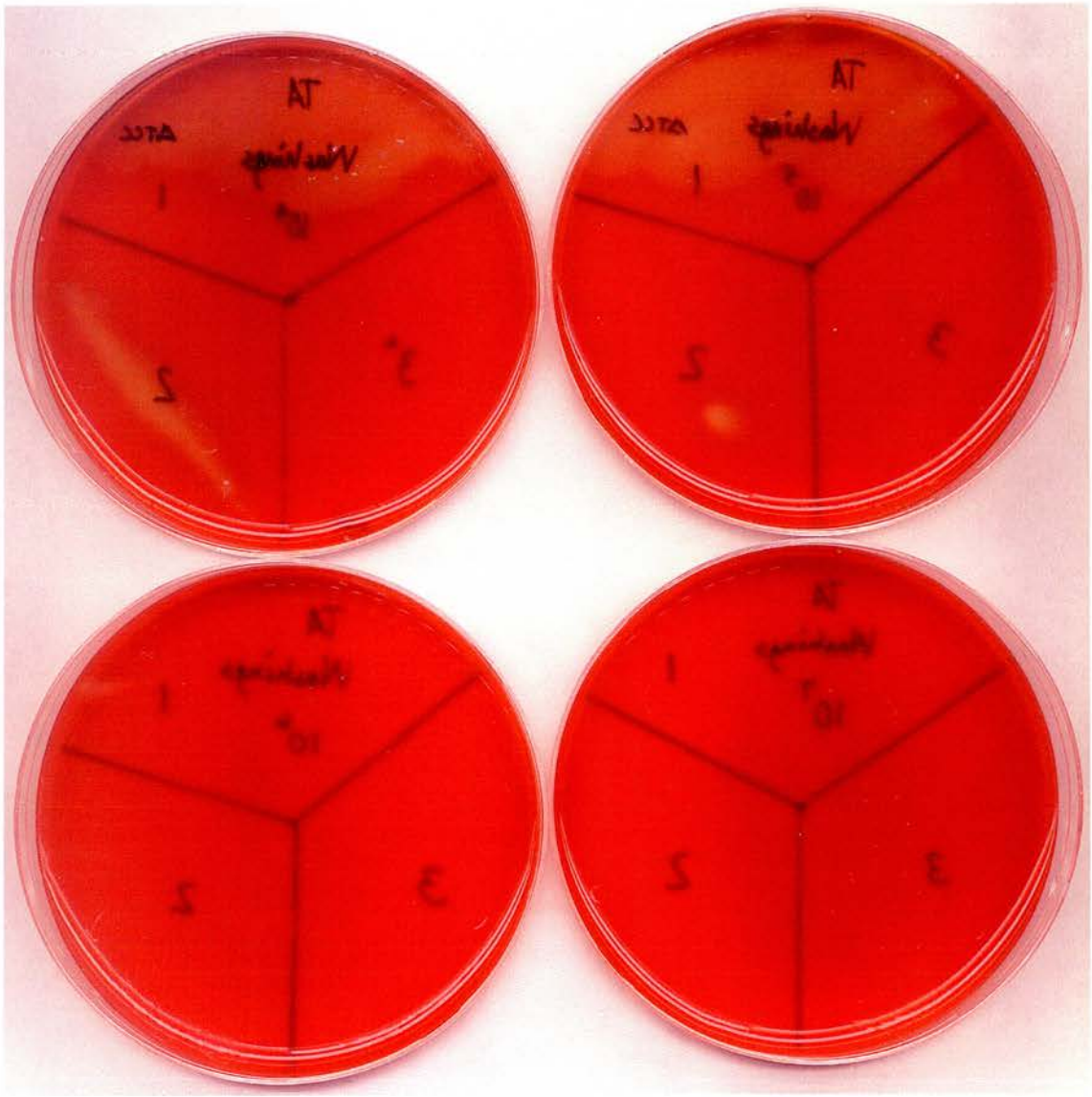

Fig. 3.11. Recovery of *B pilosicoli* on <u>TA plates</u> (incubated at 42 C) form the three washings of the IMS direct method. Inocula taken from a bacterial suspension containing 3.3 x 10^8 viable cells of *B. pilosicoli* per ml.

The IMS product of the IMS assay using *B. pilosicoli* ATCC-51139 and Dynabeads M-280 coated with PAb had a sensitivity of detection on TA plates at the level of dilution 10^1 corresponding to 5.7 x 10^8 total count of viable bacterial cells. The control (without IMS) was three 10-fold dilutions more sensitive than the IMS product to recover *B.*

pilosicoli on TA plates since the level of detection was at dilution 10^4 corresponding to 5.7×10^5 bacterial cells of *B. pilosicoli* (Fig. 3.12).

The sensitivity of the IMS product using the PAb (detection sensitivity 5.7×10^8 *B. pilosicoli* cells) was three and five 10-fold dilutions lower as compared to that of the IMS direct method and the indirect method using the MAb BJL/AC1 (level of detection 4.8×10^5 and 1.5×10^3 of *B. pilosicoli* cells, respectively) inoculated onto TA plates (Table 3.7).

Table 3.7. IMS direct method using PAb for detection of *B. pilosicoli* and *B. hyodysenteriae* in spiked pig faeces.

Dilution	Culture medium TA					
	B. pilosicoli			*B. hyodysenteriae*		
	Total count of viable bacterial cells	IMS	no IMS	Total count of viable bacterial cells	IMS	No IMS
$*10^1$	5.7×10^8	Growth no mixed (2/2)	Growth no mixed (2/2)	2.4×10^8	Growth no mixed (2/2)	Growth no mixed (2/2)
$*10^2$	5.7×10^7	NG	Growth no mixed (2/2)	2.4×10^7	Growth no mixed (2/2)	Growth no mixed (2/2)
$*10^3$	5.7×10^6	NG	Growth no mixed (2/2)	2.4×10^6	Growth no mixed (2/2)	Growth no mixed (2/2)
$*10^4$	5.7×10^5	NG	Growth no mixed (2/2)	2.4×10^5	NG	Growth no mixed (2/2)
$*10^5$	5.7×10^4	NG	NG	2.4×10^4	NG	NG
$*10^6$	5.7×10^3	NG	NG	2.4×10^3	NG	NG
$*10^7$	5.7×10^3	NG	NG	2.4×10^2	NG	NG
$*10^8$	5.7×10^1	NG	NG	2.4×10^1	NG	NG

*10-fold dilutions were made from a bacterial suspension containing 1.9×10^{10} per ml of viable cells of *B. pilosicoli* or 8×10^9 viable cells of *B. hyodysenteriae*; Growth but mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* mixed with normal intestinal bacteria; Growth no mixed: characteristic growth of *B.pilosicoli* or *B. hyodysenteriae* only; NG: no growth observed on plates

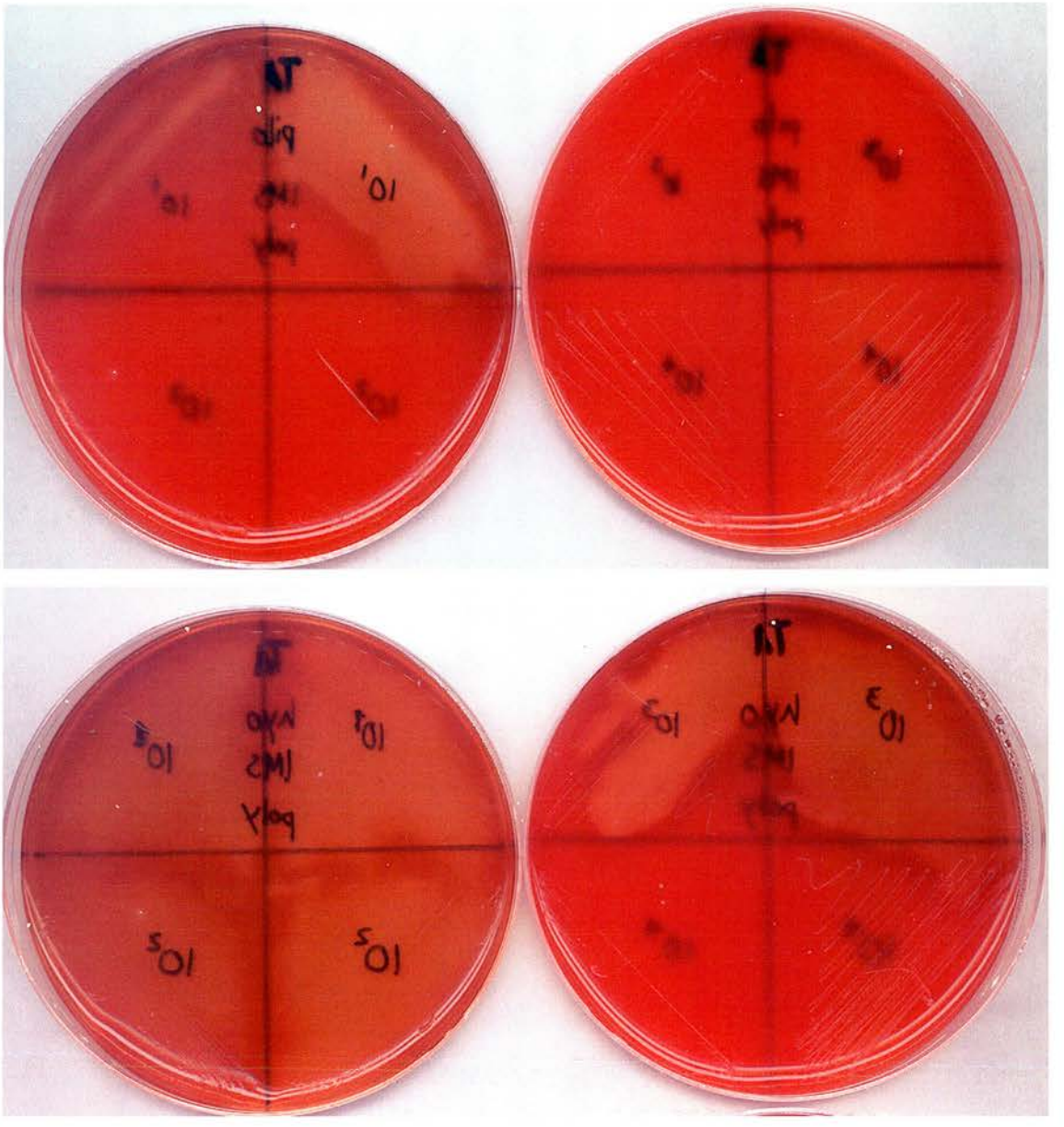

Fig. 3.12. Growth of *B. pilosicoli* (top) and *B. hyodysenteriae* (bottom) from IMS direct method using polyclonal Absspecies specific to *B. hyodysenteriae*) on <u>TA plates</u> incubated at 42 C. Inocula taken from a bacterial suspension containing 1.9 x 10^{10} viable cells of *B. pilosicoli* per ml, or 8 x 10^{9} viable cells of *B. hyodysenteriae* per ml. IMS (using polycolnal Abs) for *B. hyodysenteriae* was more sensitive than IMS for *B. pilosicoli.*

IMS indirect method for B. pilosicoli on TA plates

The IMS product of the IMS assay using *B. pilosicoli* ATCC 51139, MAb BJL/AC1 and Dynabeads M-450 was three 10-fold dilutions more sensitive for recovering *B. pilosicoli* on TA plates than the IMS product inocula on BA plates. The characteristic growth of *B. pilosicoli* developed at the level of dilution 10^5, corresponding to 1.5 x 10^3 total viable count of bacterial cells. The control (without IMS) was one 10-fold dilution more sensitive for recovering *B. pilosicoli* than that of the IMS product (indirect method), as the growth of *B. pilosicoli* developed at the level of dilution 10^6, corresponding to 1.5 x 10^2 total viable count of bacterial cells (Table 3.3. and Fig. 3.10).

IMS direct method for B. hyodysenteriae on TA plates

The IMS product of the assay using *B. hyodysenteriae* isolate P944/14 and Dynabeads M-450 coated with the MAb BJL/SH1 had a relative sensitivity at the level of dilution 10^2, corresponding to 2.5 x 10^4 total count of viable bacterial cells of *B. hyodysenteriae* isolate P944/14 which developed the characteristic growth. The control (without IMS) inocula was three 10-fold dilutions more sensitive for recovering *B. hyodysenteriae* on TA plates than that of the IMS product. The characteristic growth of *B. hyodysenteriae* developed at the level of dilution 10^4, corresponding to 2.5 x 10^1 total count of viable bacterial cells of *B. hyodysenteriae* isolate P944/14 (Table 3.4 and Fig. 3.13).
The IMS product of the assay using *B. hyodysenteriae* isolate P944/14 and Dynabeads M-280 coated with PAb had a sensitivity of detection on TA plates at the level of dilution 10^3 corresponding to 2.4 x 10^6 total count of viable bacterial cells. The control (without IMS) for the assay using PAb was two 10-fold dilutions more sensitive than the IMS product to recover *B. hyodysenteriae* since the level of detection was at dilution 10^4, corresponding to 2.4 x 10^5 bacterial cells of *B. hyodysenteriae* (Fig. 3.12).

The sensitivity of the IMS using the PAb (detection sensitivity 2.4 x 10^6 *B. hyodysenteriae* cells) was one and two 10-fold dilutions lower than the IMS direct method and IMS indirect method using the MAb BJL/SH1 (level of detection 2.2 x 10^4

and 9.9 x 10^3 of *B. hyodysenteriae* cells, respectively) inoculated onto TA plates (Table 3.7 and Fig. 3.13).

IMS indirect method for B. hyodysenteriae on TA plates

The IMS product of the assay using *B. hyodysenteriae* isolate P944/14, MAb BJL/SH1 and Dynabeads M-450 was one 10-fold dilution more sensitive for recovering *B. hyodysenteriae* on TA plates than that of the IMS direct method. The characteristic growth developed at the level of dilution 10^3, corresponding to 9.9 x 10^3 total count of viable bacterial cells of *B. hyodysenteriae* isolate P944/14. The control (without IMS) inocula was three 10-fold dilutions more sensitive than the IMS, the characteristic growth of *B. hyodysenteriae* developed at the level of dilution 10^5 corresponding to 9.9 total count of viable bacterial cells of *B. hyodysenteriae* isolate P944/14 (Table 3.5 and Fig. 3.13).

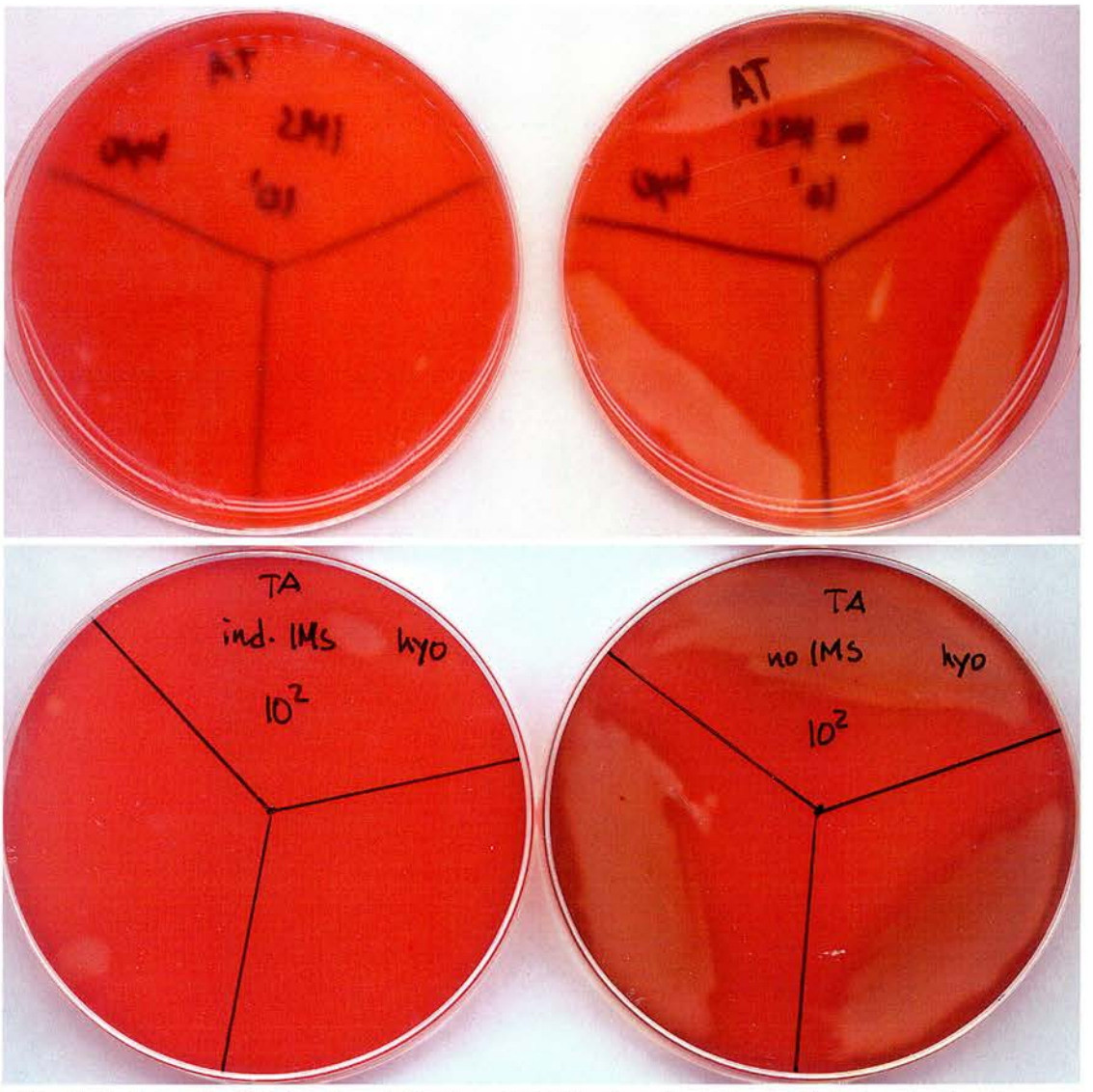

Fig. 3.13. Growth of *B. hyodysenteriae* from IMS direct method (top, left), indirect method (bottom, left), and controls without IMS (top and bottom right) on TA plates, showing pure culture. Top, inocula taken from dilution 10^1 of a bacterial suspension containing 7.5 x 10^6 viable cells of *B. hyodysenteriae* per ml. Bottom, inocula taken from dilution 10^2 of a bacterial suspension containing 3.3 x 10^7 viable cells of *B. hyodysenteriae* per ml.

Comparison of PCR with bacteriological culture of B. pilosicoli ATCC-51139 and B. hyodysenteriae isolate P944/14 on TA plates

The relative sensitivity of the PCR on spiked faeces (without IMS) samples was at the level of dilution 10^4 (4.8×10^4 *B. pilosicoli* ATCC-51139 cells) which was one 10-fold dilution more sensitive than the PCR performed on the IMS product obtained by direct method. However, the relative sensitivity of the PCR was two 10-fold dilutions lower than the bacteriological culture of the control (without IMS) since 2 out of 3 inocula developed the characteristic growth of *B. pilosicoli* on TA plates. Despite the bacteriological culture of the IMS for *B. pilosicoli* (indirect method) had higher sensitivity (1.5×10^3) for recovering *B. pilosicoli* ATCC-51139 on TA plates than the IMS (direct method, 4.8×10^5). The PCR from samples of both (direct and indirect) methods had similar sensitivity as detection was at the level of detection was 4.8×10^5 cells of *B. pilosicoli* ATCC-51139 and 1.5×10^5 cells of *B. pilosicoli* ATCC-51139, respectively (Tables 3.2 and 3.3, and Fig. 3.14a and 3.14b). On the other hand, PCR of the IMS (direct method without washing) was one 10-fold dilution more sensitive for detecting *B. pilosicoli* ATCC-51139 than normal IMS performed with washings (Table 3.6). That correlated with the improvement of one 10-fold dilution to recover *B. pilosicoli* on culture by the IMS without washing. However, bacteriological culture of *B. pilosicoli* ATCC-51139 on TA plates was two 10-fold dilutions more sensitive than PCR.

The PCR of the IMS (direct method for *B. hyodysenteriae* isolate P944/14) sample was one 10-fold dilution more sensitive for detection of *B. hyodysenteriae* than the bacteriological culture of the IMS (direct method) product on TA plates. The PCR from control (without IMS) samples had the same sensitivity of detection as the bacteriological culture on TA plates, which was at the level of dilution 10^4 corresponding to 2.5×10^1 total count of viable bacterial cells (Table 3.4).

PCR was more sensitive for detecting both *B. pilosicoli* ATCC-51139 and *B. hyodysenteriae* isolate P944/14 on control (without IMS) samples than from IMS (direct

or indirect method) samples, and also more sensitive on the IMS (without washings) samples than from the IMS normally performed samples. Interestingly, PCR was more sensitive for detecting *B. hyodysenteriae* on control without IMS (detection level 2.5 x 10^1 bacterial cells) than *B. pilosicoli* on control (without IMS) and IMS (without washing) samples, detection level 4.8 x 10^4 or 9.9 x 10^3 bacterial cells, respectively.

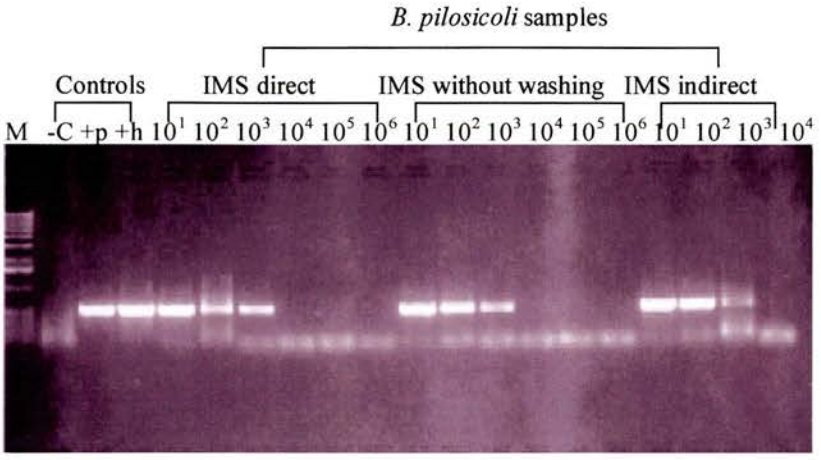

Fig. 3.14a. Agarose gel displaying a characteristic 555 bp PCR product of *Brachyspira* species generated from IMS (for *B. pilosicoli*) samples. M, DNA ladder 1 KB (0.5-10 kb).

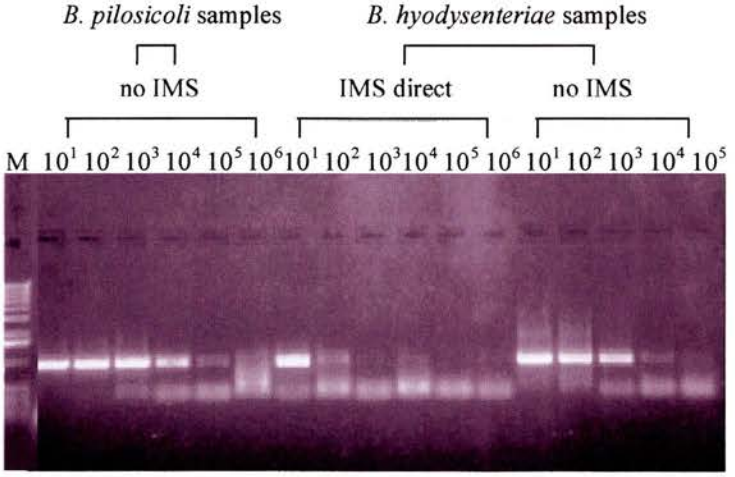

Fig. 3.14b Agarose gel displaying the characteristic 555 bp PCR product of *Brachyspira* species generated from IMS and controls (without IMS) samples. M, DNA ladder 1 KB (0.5-10 kb)

Enzymatic digestion of PCR products

The enzymatic digestions of the PCR products of DNA from *B. pilosicoli* ATCC-51139 generated a banding pattern characterised by three well defined bands of 472, 57 and 29 bp molecular size. Whereas the enzymatic digestion of DNA products from *B. hyodysenteriae* isolate P944/14 generated a banding pattern characterised by bands of molecular sizes 298, 201 and 51 bp, as displayed on polyacrylamide gels and silver stained (Fig. 3.15). Although this work was carried out using known species of *Brachyspira*, the enzymatic digestion was performed to confirm the absence of any other *Brachyspira* species that could be unexpectedly present and could have an effect on the sensitivity or specificity of MAbs, IMS or PCR results.

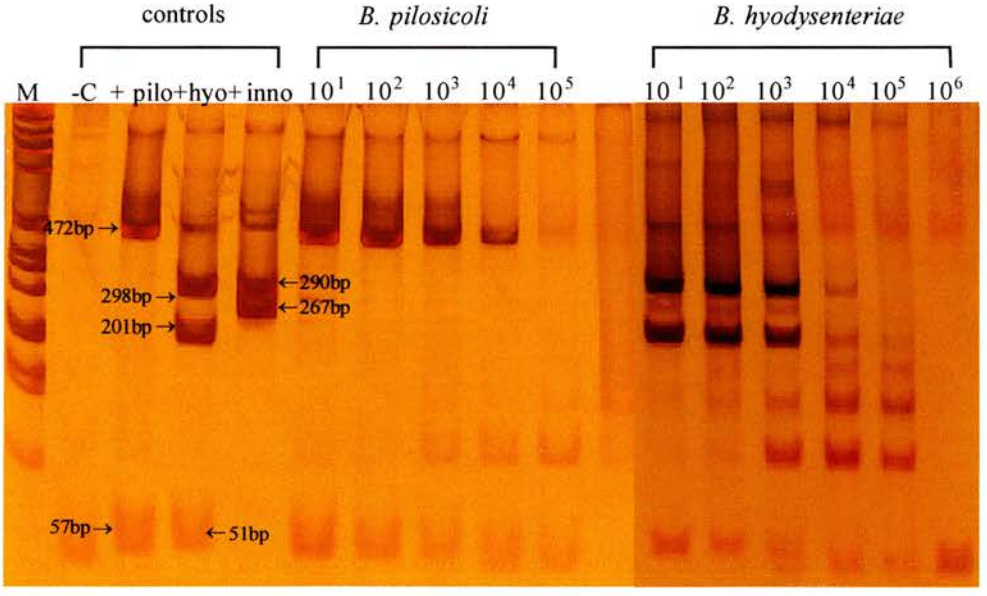

Fig. 3.15. Enzymatic digestion of PCR products from *B. pilosicoli* and *B. hyodysenteriae* IMS samples. The +ve controls show the characteristic DNA bands for *B. pilosicoli*, *B. hyodysenteriae* and *B. innocens*. Sample lanes showing the dilution of the bacterial suspension used to spike pigs faeces for IMS assays, and that no other spirochaete species was present in the IMS samples than *B. pilosicoli* and *B. hyodysenteriae*, respectively.

Discussion

In this study an IMS technique was developed using MAbs to *B. pilosicoli* and to *B. hyodysenteriae*, and a PAb to *B. hyodysenteriae*. Assays to determine titre, specificity and efficiency of the MAbs and the PAb were carried out. The antibody titres found for both MAbs were 1:160. A dilution 1:250 of the MAb BJL/AC1 has been used for the identification of the Ag protein of size 29 kDa located on the membrane of *B. pilosicoli*, which had ≤38.8 % amino acid homology to a pyruvate-ferredoxin oxidoreductase (Rayment *et al,* 1998). That result indicates that higher dilutions of the MAb are still effective for recognising the Ag protein of *B. pilosicoli*. Surprisingly, the MAb BJL/AC1 showed a weak reaction towards dot blot preparations from two isolates of both *B. innocens* and *B. hyodysenteriae* in specificity assays (data not shown). Contrary to these results, Lee and Hampson (1995) found high specificity of MAb BJL/AC1 since no reaction was observed to cell envelope preparations of five other species of intestinal spirochaetes. The present study showed that MAb BJL/SH1 did not react towards isolates of *B. pilosicoli* or *B. innocens*, indicating higher specificity than the MAb BJL/AC1. As for MAb BJL/SH1 current results were similar to those reported by Lee and Hampson (1996) in which this MAb only and exclusively reacted with the cell envelope preparations of *B. hyodysenteriae*, specifically with a 30 kDa Ag protein. The Ag protein of size 29 kDa of *B. pilosicoli* isolates from pigs, dogs and humans, and the Ag protein of size 30 kDa of *B. hyodysenteriae* isolates from pigs, mouse and chicken, have been properly identified using the correspondent BJL/AC1 and BJL/SH1, respectively (Rayment *et al,* 1997).

The specificity of the PAb raised in rabbits against *B. hyodysenteriae* was not high, this antiserum showed cross-reaction with the cell envelope protein preparations of various isolates of *B. pilosicoli* and *B. innocens*. These results were not surprising for a polyclonal antiserum for *Brachyspira* species since cross-reaction between *B. hyodysenteriae* and *B. innocens* has been reported (Joens and Marquez, 1986; Chatfield *et al,* 1988; Miller *et al,* 1988; Smith *et al,* 1990). This also supports studies by Lee and

Hampson (1995 and 1996), in which a polyclonal mouse antiserum showed cross-reaction with isolates of five different species of intestinal spirochaetes of pig and human origin.

In the assays for specificity and Western blotting, it was observed that an Ag protein of approximately 50 kDa was recognised by goat and swine immunoglobulins conjugated with horseradish peroxidase used as secondary antibody in several *B. pilosicoli* isolates, namely the two dog isolate K19 and K22, and the porcine isolates P32/2/97, P93/2/94, P595/3/00, P657/8/00 and P676/2/00. Interestingly, those immunoglobulins did not recognise Ag proteins from isolates of *B. innocens* nor *B. hyodysenteriae*. The studies by Lee and Hampson (1995 and 1996) described the methodology to develop the MAbs BJL/AC1 and BJL/SH1 in which SDS-PAGE and Western blotting (among other techniques to verify the specificity of that MAb) were used with Goat anti-Mouse as a second Ab. In those studies the recognition of cell envelope protein preparations of *B. pilosicoli* isolates from pigs and humans by the second antibody (goat immunoglobulins conjugated with horseradish peroxidase) was not reported. Although controls were included, in that study the controls for first Ab were not mentioned. In the present study, Western blotting assays showed recognition of the cell envelope preparations of *B. pilosicoli* isolates from pigs and dogs by goat and swine immunoglobulins, and the reaction remained positive using a different batch of reagent. A possible explanation could be intrinsic production of peroxidase by *B. pilosicoli*, however, the controls for Ag preparations with only developer added showed no reaction, indicating no intrinsic peroxidase production by *B. pilosicoli*. Although, the goat immunoglobulins recognised that Ag in cell envelope protein preparations of field isolates that had not been characterised and had not been used in other studies, the cell envelope proteins of *B. pilosicoli* type strain ATCC-51139 were not recognised by the goat immunoglobulins. In the studies by Lee and Hampson (1995 and 1996), Goat anti-Mouse was also used as a second antibody but the manufacturer was not the same as the one used in the present study; hence, it could be that that particular animal in which the antiserum was raised had developed Abs against certain isolates of *B. pilosicoli*. Alternatively, *B. pilosicoli*

may contain an immunoglobulin binding protein, however, further studies would be required to confirmed this.

It is known that *Brachyspira* species are fastidious organisms for their growth under laboratory conditions. Selective media have been developed to enhance the isolation of these micro-organisms from faecal samples and IMS may further enhance sensitivity. Growth of *B. pilosicoli* and *B. hyodysenteriae* after IMS assays was assessed by plating out onto both conventional as BA and selective culture media as TA (containing the antibiotics spectinomycin, vancomycin and colistin). In general, the inocula from the IMS and those of controls without IMS produced growth of *B. pilosicoli* and *B. hyodysenteriae* mixed with bacteria of the normal pig intestinal flora when inoculated onto BA plates. In contrast, the aliquots of the same inocula onto TA plates produced pure cultures of each *B. pilosicoli* and *B. hyodysenteriae* as confirmed by microscopic examination of smear preparations. Selective culture media plates were superior in terms of the growth and significantly reduced the faecal flora by action of the antibiotics added. Selective media developed by Jenkinson and Wingar (1981); Kunkle and Kinyon (1988) and more recently Calderaro *et al* (2001) have demonstrated that those media were more sensitive for the isolation of *B. hyodysenteriae* than conventional media, giving higher probability of recovering this micro-organism in pure culture.

Although BA and TA plates incubated at 37°C produced growth of *B. pilosicoli* and *B. hyodysenteriae,* better quality of growth and higher sensitivity for isolation was obtained when BA and TA plates were incubated at 42 °C. The growth at 42 °C was heavier and more consistent compared to the growth at 37 °C. Incubation temperatures for intestinal spirochaetes have been reported to be in the range of 37 to 42 °C (Jensen, 1997). High sensitivity for the isolation of *B. pilosicoli* strain ATCC-51139 has been achieved by incubation of selective plates at 42 °C on which an inoculum of 15 cells of *B. pilosicoli* was recover after 7 days of incubation (Fellström *et al,* 1997), and as few as 2 cells of *B. hyodysenteriae* (Fellström *et al,* 2001). In the current study the incubation period allowed for the IMS plates was up to 4 days. Higher sensitivity might have been

achieved if the incubation period had been longer, however, length of incubation was not evaluated.

Unexpectedly, higher sensitivities were obtained by control (without IMS) inocula for recovering *B. pilosicoli* and *B. hyodysenteriae* on culture on TA plates incubated at 42 °C, as compared to the inocula of the IMS when performed with the recommended washings. Although, inocula of the IMS with washings detected the target cells its sensitivity was still lower than direct culture. The level of sensitivity of the IMS for the isolation of *B. pilosicoli* and *B. hyodysenteriae* from faeces in the current study may be enough to detect these micro-organisms in clinically affected animals since the excretion level of experimentally infected gnotobiotic pigs with *B. hyodysenteriae* and *B. pilosicoli* has been reported at the level of 1×10^5 to 2×10^9 bacterial cells per g of faeces (Neef *et al,* 1994), and at the level of 10^7 to 10^8 bacterial cells per g of mouse faeces (Joens, 1980). However, that level of sensitivity may not be adequate for the detection of low numbers of bacteria excreted by carrier animals.

Direct culture of *B. pilosicoli* was three 10-fold dilutions more sensitive than IMS (direct method) and one 10-fold dilution than IMS (indirect method), and direct culture of *B. hyodysenteriae* was three 10-fold dilutions more sensitive than IMS (direct method) and two 10-fold dilutions more sensitive than IMS (indirect method). A study by Osaki *et al* (1998) also reported that direct culture of an intestinal pathogen of helical form such as *Helicobacter pylori* was more sensitive than IMS using a purified IgG polyclonal Ab to coat the magnetic beads. One of the possible explanations for lower sensitivity rate by IMS could be that manipulation of *Brachyspira* cells during the IMS procedure (washings) might affect their viability due to exposure to aeration during pipetting, although *Brachyspira* species have been reported to be aerotolerant (Baseman *et al*, 1990).

In view of the effects of manipulations of *Brachyspira* cells during the washing steps of IMS and considering the effectiveness of the antibiotics supplemented to the selective media (TA plates), IMS assays without washing were also carried out to determine

whether that factor (washing) was influencing the sensitivity of IMS. The results of the IMS direct method for *B. pilosicoli* without washing increased the sensitivity rate of recovering the target cells by two 10-fold dilutions. Moreover, *Brachyspira* cells were recovered from the supernatant of the washings, indicating that target cells were indeed washed off during the washing steps. With the objective that IMS would improve detection of *B. pilosicoli* and *B. hyodysenteriae* in faecal samples from carrier pigs shedding low numbers of bacteria, the IMS without washing was thought to increase the efficiency of the IMS to recover *Brachyspira* cells. On the other hand, the washing of the IMS product becomes crucial when further tests such as PCR are going to be applied on the IMS product recovered from faecal samples since IMS product without washing would contain more PCR inhibitory factors which may influence its sensitivity (Lou *et al,* 1997). However, in this study such factors did not seem to affect the sensitivity of the PCR assay for detection of *B. pilosicoli* since the sensitivity of the PCR on samples without washing was higher (9.9×10^3) than that of IMS samples performed with washings (4.8×10^4). It has been reported that PCR can be influenced by components of faeces such as bile acids, bilirubin or polysaccharides which interfere with the efficiency of *Taq* polymerase (Panaccio and Lew, 1997). A study on detection of *H. pylori* using IMS followed by PCR of the IMS product of spiked faeces with and without washing found that PCR detected the target bacterial cells at the level of 10^7 per ml when washing was applied to the IMS product. Comparatively, the IMS product without washing contained more target cells (10^8) per ml, but these numbers of target bacterial cells were not detected by PCR. However, PCR detected *H. pylori* at the level of 10^5 to 10^6 cells per ml from bacterial suspension samples without faeces, indicating that the sensitivity of PCR might have been negatively affected by the presence of inhibitor substances present on faeces (Engroth and Engstrand, 1995).

From the results of the IMS without washing in which higher sensitivity was obtained for the isolation of *B. pilosicoli*, two possible explanations may be given. Firstly, target cells might have been released from the MAb bound to the Rat anti-Mouse antibody (precoated on the Dynabeads M-450); secondly, both the MAb binding the target cells

might released from the Rat anti-Mouse, leaving the magnetic beads without captured product as a result of an unstable antigen-antibody (Ag-Ab) reaction. Though the coating rate obtained for both MAbs BJL/AC1 and BJL/SH1 gave a positive indication of the affinity of the Rat anti-Mouse to the mouse MAbs (IgM class), though, the stability of the Ab-Ab and Ag-Ab reactions was not evaluated.

It has been documented that bacteria in general can be non-specifically adsorbed to solid surfaces in the presence of electrolytes (Marshall *et al,* 1971), which could explain the lower sensitivity of capturing the target cells by the IMS obtained in this study. Bacteria of the normal flora might have been adsorbed to the surface of the magnetic beads obstructing the receptors for the MAb despite the use of a blocking agent (bovine serum albumin) in the washing buffer, as recommended by the manufacturer. A method to reduce non-specific adsorption of undesired bacteria in IMS assays has been suggested, consisting of low ionic strength buffer treated with a chelating agent (Tomayasu, 1998). That method increased the sensitivity of IMS from 9.6 to 31.4 % as compared to conventional IMS without low ionic strength buffer.

Another factor that might contributed to the sensitivity of IMS applied to intestinal spirochaetes was the morphology of the target cells (*B. pilosicoli* 5.2-11.0 μm length and *B. hyodysenteriae* 5.9-12.9 μm length) which could be double the size of the Dynabeads M-450 (4.5 μm diameter). However, IMS has also been used for the isolation of the spirochaete *Leptospira borgpetersenii* from urine samples. The sensitivity of detection was at the level of 10^1 bacterial cells, demonstrating the high sensitivity of IMS for capturing spirochetal bacteria using even smaller magnetic beads, Dynabeads M-280 2.8 μm diameter (Taylor *et al,* 1997). It is likely that the low numbers of other bacteria in urine contributed to the high sensitivity in that study. In agreement with this study, in that study IMS followed by PCR was not superior to direct culture to recover the target cells from urine since 77.0 % (21/27) of the samples were culture positive compared to 55.0 % (15/27) of the samples that gave PCR positive. The sensitivity of IMS followed by PCR in the study by Taylor *et al* (1997) was higher than the highest PCR sensitivity

obtained in this study which was at the level of 2.5 x 10^1 bacterial cells of *B. hyodysenteriae* from the control (no IMS) samples.

In the present study, the sensitivity of the PCR for detection of *B. pilosicoli* was at the level of 9.9 x 10^3 bacterial cells. Sensitivities of PCR for various species of intestinal spirochaetes have been reported elsewhere and are consistent with the PCR sensitivity of the present study. For instance, a PCR using the primers SF1 and SR1 flanking a region on the 23S rDNA gene of *B. hyodysenteriae, 'Brachyspira-Serpulina' intermedia* and *B. pilosicoli* had a sensitivity detection at the level of 5 x 10^5 cells per gram of faeces (Leser *et al,* 1997). Another PCR developed to amplify a sequence of the DNA of *B. hyodysenteriae* had a sensitivity detection rate at the level of 10^3 to 10^4 bacterial cells per 0.2 g of faeces (Atyeo *et al,* 1998). That PCR had higher sensitivity detection rate since 83.3 % (15/18) of samples from naturally infected pigs with *B. hyodysenteriae* gave positive by PCR compared to 50.0 % (9/18) positive by direct culture (Atyeo *et al,* 1998). Similar sensitivity was found with another PCR to amplify a region of the 16S rRNA gene of *B. pilosicoli,* which had a sensitivity of detection at the level of 10^4 bacterial cells per 0.2 g of faeces. That PCR was also found to be more sensitive (60.0 %, 27/45) as compared with a direct culture (24.4 %, 11/45) for detecting positive samples from naturally infected pigs with *B. pilosicoli* (Atyeo *et al,* 1998), however, more sensitive PCR assays have also been reported. For instance, Park *et al* (1995) developed a highly sensitive and specific PCR for *B. pilosicoli* using two primers targeting sequences of the 16S rRNA. One of the primers has been designated as Ac1, which consisted of a unique sequence on the 16S rRNA gene of *B. pilosicoli.* That PCR had a sensitivity detection level of 250 cells per reaction (Park *et al,* 1995).

Another PCR developed by Fellström *et al* (1997) detected as few as 24 *Brachyspira* cells of the reference strain P43/6/78 from bacteriological culture, however, that PCR failed to detect this strain in particular from spiked pig faeces. Interestingly, the recovery rate of the reference strain P43/6/78 on culture was at the level of 15 cells per inoculum of spiked faeces when incubated at 42 °C for 7 days as on selective medium

containing antibiotics (see above Fellström *et al,* 1997). In the current study the highest sensitivity to recover *B. pilosicoli* type strain ATCC-51139 from spiked faeces on selective medium by direct culture was at the level of 1.5×10^2 total bacterial cells when incubated at 42 °C. That level of sensitivity was just one 10-fold higher than that (15 cells) reported by Fellström *et al* (1997) for the recovery of *B. pilosicoli* on selective medium. It has been suggested that a better approach for a quick diagnosis of intestinal spirochaetes with particular emphasis on *B. pilosicoli* would be by the isolation on selective medium coupled with PCR rather than the approach of culture and biochemical tests (Fellström *et al,* 1997). However, for laboratories lacking of molecular facilities the recommended method of detection of intestinal spirochaetes is bacterial culture and biochemical reactions including the type of haemolysis, indole production, hippurate hydrolysis and activities for α- and β- glucosidase, and α-galactosidase (Hommez *et al,* 1998). Accurate identification of intestinal spirochaetes may need further investigations since atypical biochemical reactions occur as result of mixed infections.

In this study the isolation of *B. pilosicoli* and *B. hyodysenteriae* was compared using IMS (magnetic beads coated with MAb specific for each bacterium species) samples and the respective control samples. Also the sensitivity of a PCR assay was compared to detection of these bacteria by direct culture. The current study showed that selective culture medium was superior to the conventional culture medium for the isolation of these micro-organisms since the cultures were practically pure. The use of selective culture media could save labour and time for obtaining a pure culture in the first instance. An incubation temperature of 42 °C rather than 37 °C favoured the growth of both *B. pilosicoli* and *B. hyodysenteriae*. Under the conditions used in this study the isolation of *B. pilosicoli* and *B. hyodysenteriae* by direct culture was more sensitive than the IMS performed with recommended washings. Therefore the successful detection of *Brachyspira* cells excreted in low numbers by carrier animals using IMS will require further development of this technique to increase its sensitivity. However, IMS for the isolation of *B. pilosicoli* (performed without washings) had higher sensitivity than direct

culture showing potential of this technique for detection of porcine intestinal spirochaetes.

Direct culture of *B. pilosicoli* on selective culture medium was also slightly more sensitive than PCR for detection of this bacterium and as for *B. hyodysenteriae* direct culture was as sensitive as PCR. However, it has to be considered that the bacterial strains used in this study were adapted to laboratory conditions (high passage) and freshly grown so, it might be that those factors contributed to their high culture success rate. Therefore, further studies are required in order to evaluate and compare these methods of detection of *Brachyspira* cells from clinical samples. An advantage of direct culture being more sensitive than IMS and equally sensitive as PCR is that it would reduce cost and it may be the approach to be considered for isolation of *B. pilosicoli* and *B. hyodysenteriae* in the first instance. However, the effect on viability of bacteria due to the delay of samples reaching the laboratory or inappropriate submission (samples over heated) needs to be considered when attempting isolation from clinical samples. On the other hand, on the grounds of clinical diagnosis, the speed of results is demanded and PCR is invaluable in this regard. Considering the potential sensitivity of PCR, a possible way to treat clinical samples could be IMS (without washing) followed by PCR.

In conclusion, IMS normally performed (with the respective washings) was less sensitive than direct culture on TA plates whereas IMS for *B. pilosicoli* without washing was highly sensitive and its level of detection could be compared to that of direct culture and PCR. The IMS without washing showed potential for detection of porcine intestinal spirochaetes, however, more studies need to be done to develop a method that avoids significant lost of target cells since it was demonstrated that target cells were washed off with washings. For laboratory practical terms, the combination of TA plates and incubation temperature at 42 °C proved to be sensitive enough for the isolation of the porcine intestinal spirochaetes *B. pilosicoli* and *B. hyodysenteriae* from faecal samples under the conditions evaluated in this study. Application of IMS to clinical samples may increase the cost and labour.

Chapter four

GENETIC DIVERSITY AMONG FIELD ISOLATES OF *Brachyspira pilosicoli*.

Introduction

The genus *Brachyspira* includes commensal and pathogenic species among which *B. pilosicoli* and *B. hyodysenteriae* are pathogenic for pigs. *B. pilosicoli* has also been associated with disease in humans (Duhamel *et al*, 1995a; Koopman *et al*, 1993; Trott *et al*, 1996; Park *et al*, 1995) and other animals (Cowley and Hill, 1986; Davelaar *et al*, 1986; Songer *et al*, 1978; Lee and Hampson 1994). Due to the wide host range of *B. pilosicoli* there is need to type isolates for epidemiological purposes. A recent study in the UK on the causes of porcine colitis found *B. pilosicoli* being involved in 25.0 % of outbreaks as the only pathogen, and in a further 27.0 % of outbreaks as a co-pathogen (Thomson *et al,* 1998), similar figures have are reported in chapter 2 of this study. Since pigs may be colonised by other *Brachyspira* species as commensals, identification of species and typification of outbreak isolates is important for epidemiological differentiation.

Differentiation of species within the genus *Brachyspira* has been done by biochemical, phenotypic and genomic examinations. Phenotypic methods include serotyping lipopolysaccharides (LPS) antigens (Baum and Jones, 1979; Hampson, 1990; Li *et al*, 1991). Biochemically the porcine intestinal spirochetes have been classified into four different groups (I-IV) based on indole production, hippurate hydrolysis and activity of α and β-glucosidase, and α-galactosidase (Fellström and Gunnarson, 1995), with such classification placing *B. pilosicoli* into group IV.

Molecular comparisons for several bacteria have been made on the basis of genomic similarities. In DNA:DNA comparisons, a DNA homology with values between 60 and 100 % indicate that two strains belong to the same species (Holdeman *et al,* 1984). In sequence comparisons of the 16S rRNA gene, a level of 95.0 to 97.0 % indicates that two species of the same genus are different (Paster and Dewhirst 1988). Based on these parameters, it has been proposed that the genus *Brachyspira* can be differentiated from

other genera of spirochaetes by the values of DNA sequence homology and sequence similarity of the 16S rRNA gene (Stanton *et al*, 1991).

DNA fingerprint analysis by arbitrarily primed-polymerase chain reaction (AP-PCR) of WBHIS associated with PCS (*B. pilosicoli* including the type strain P43/6/78) showed that isolates from different countries i.e. US, UK and Australia, clustered together in a branch and were separated from the type strain B78 of *B. hyodysenteriae* on a phylogenetic tree generated using the random primer 5'GGAAACAGCTATCACCATGA 3' (Duhamel *et al,* 1995a).
The genome of *B. hyodysenteriae* has been explored for at interspecies and intraspecies variation using AP-PCR/RAPD for epidemiological purposes (Dugourd *et al,* 1996). PCR using arbitrary primers have also been used to screen the DNA of other spirochaetes such as *Borrelia burgdorferi* to delineate genetic groups (Welsh *et al,* 1992).

The various molecular typing methods have confirmed the diversity of intestinal spirochaetes and have also given the bases for characterisation and taxonomic classification of emerging species. Among these methods, arbitrarily primed PCR (AP-PCR) or random amplification of polymorphic DNA (RAPD) generates reproducible DNA fingerprints to distinguish genetic variation between strains (Williams *et al*, 1990). AP-PCR/RAPD has been widely used for comparing polymorphism through the analysis of the banding patterns generated from various species of procaryotes and eucaryotes (Welsh and McClelland, 1990).

The literature supports the fact that there is genetic diversity of intestinal spirochaetes; therefore, the aim of this study was to develop an AP-PCR/RAPD technique to screen the genetic variation of single and multiple isolates of *B. pilosicoli* from single herds for epidemiological purposes.

Material and Methods

Bacterial isolates

Forty-three field isolates of *B. pilosicoli* were recovered from cases of porcine colitis arising from outbreaks on 21 separate farms in the UK (Fig 4.0), as part of a surveillance programme conducted by the Scottish Agricultural College. A numerical code and colour (e.g. P152-1) was assigned to each farm, and multiple isolates were obtained from several farms. Two dog isolates were recovered from the faeces samples of dogs with diarrhoea submitted for diagnosis to the Veterinary Science Division of the SAC. One human isolate kindly provided by Dr. Anne Livesley (Aston University, UK). The type strain ATCC-51139 was obtained from the American Tissue Culture Collection and the *B. pilosicoli* reference strain M1 for the UK was of obtained from the Veterinary Laboratories Agency (VLA), Winchester, UK. Also one field isolate of *B. hyodysenteriae* was included in this study as an outgroup. All isolates are listed in Table 4.1.

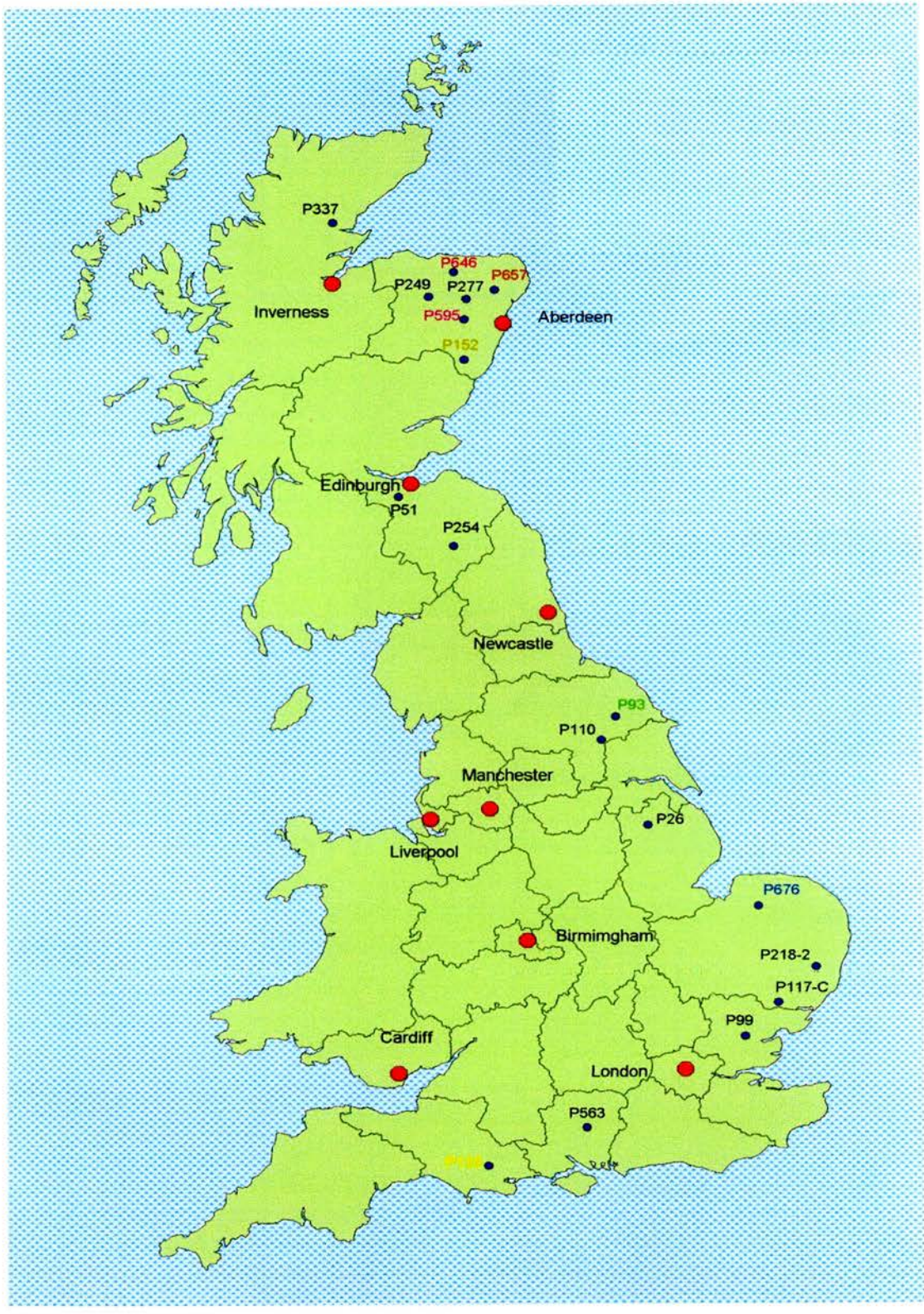

Fig. 4.0. Geographic distribution of pig farms from which *B. pilosicoli* was isolated of samples submitted to the SAC.

Table 4.1. Reference strains and field isolates of *B. pilosicoli* and *B. hyodysenteriae* of which DNA was used in AP-PCR analysis.

Number of isolates	*Isolate*	*Year of isolation*	*Origin*	*Species*
-	ATCC-51139 (P43/6/78)	1978	pig	*B. pilosicoli*
-	ExRDVC	Unknown	human	"
-	M1 (VLA)	Unknown	pig	"
-	K19 (M1701/99)	1999	dog	"
-	K22 (M2901/99)	1999	"	"
-	P393/8/98	1998	pig	*B. hyodysenteriae*
1	P4	1993	"	*B. pilosicoli*
1	P6	1993	"	"
1	P26	1993	"	"
1	P32	1994	"	"
1	P34	1994	"	"
1	P99/7/93	1993	"	"
1	P110/4/93	1993	"	"
1	P117-C/99	1999	"	"
1	P254/97	1997	"	"
1	P277/97	1997	"	"
1	P337/98	1998	"	"
1	P563/97	1997	"	"
1	P218/2/99	1999	"	"
2	P152/1/99	1999	"	"
	P152/8/99	"	"	"
2	P249/5/99	1999	"	"
	P249/9/99	"	"	"
3	P93/2/94	1994	"	"
	P93/3/94	"	"	"
	P93/4/94	"	"	"
4	P126/5/99	1999	"	"
	P126/7/99	"	"	"
	P126/9/99	"	"	"
	P126/12/99	"	"	"
4	P595/1/00	2000	"	"
	P595/2/00	"	"	"
	P595/3/00	"	"	"
	P595/9/00	"	"	"
4	P676/1/00	2000	"	"
	P676/2/00	"	"	"
	P676/3/00	"	"	"
	P676/4/00	"	"	"
5	P646/1/00	2000	"	"
	P646/4/00	"	"	"
	P646/6/00	"	"	"
	P646/7/00	"	"	"
	P646/8/00	"	"	"
5	P657/1/00	2000	"	"
	P657/2/00	"	"	"
	P657/3/00	"	"	"
	P657/5/00	"	"	"
	P657/8/00	"	"	"

Preparation of bacterial cells for DNA extraction

The culture product of two blood agar plates (4-day-old) was used as the inoculum for the propagation of bacterial cells in 10 ml of brain heart infusion (BHI) broth [Oxoid, UK] supplemented with 5.0 % (v/v) filter sterilised rabbit serum in tissue culture flasks (size 25 cm^2) (Corning, UK). Purity of cultures was checked by Gram stain of smears. Broth cultures were incubated anaerobically at 38 °C for 3 to 4 days. The growth in broth was centrifuged at 8,000 g for 10 minutes and washed twice with phosphate buffered saline solution (PBS). The pellet was re-suspended in PBS and calibrated by optical density (OD) between 0.6 and 0.8 at wavelength of 600 nm.

DNA extraction and quantification

The initial PCRs were conducted using DNA extracted by the phenol chloroform method (Sanbrook *et al,* 1989). Briefly, the bacterial pellets were resuspended in 567 µl of TE buffer (Appendix A), then 30 µl of 10.0 % SDS and 3 µl of 20 mg/ml proteinase K were added. The mix was vortexed briefly and incubated at 37 °C for 1 hr to degrade the bacterial cell wall, then 200 µl of 5 M NaCl was added followed by 80 µl hexadecyl trimethyl ammonium bromide (CTAB). This was mixed thoroughly to remove cell wall debris and denatured proteins and polysaccharides. After that, the tube was incubated at 65 °C for 10 minutes followed by addition of an equal volume (0.7 to 0.8 ml) of 24:1 chloroform isoamyl alcohol to remove the CTAB protein-polysaccharides complexes, then the sample was spun at 8000 g for 5 minutes. The aqueous supernatant was transferred into a clean Eppendorf tube adding an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol, then mixed thoroughly and spun at 8000 g for 5 minutes. The supernatant was transferred into a new tube followed the addition of 0.6 volumes of isopropanol to precipitate the nucleic acids. The tube was spun at 8000 g for 5 minutes, washed with 400 µl 70 % ethanol to remove residues, and then spun again at 8000 g. The ethanol supernatant was discarded and the pellet allowed to dry at room temperature. Finally, the nucleic acids were re-suspended in 50 µl TE buffer and kept at 4 °C until required. This method yielded highly variable quantities of DNA; therefore,

DNA was extracted using Qiagen columns for DNA purification (QIAamp-DNA Mini Kit, Qiagen-UK) following the manufacturer's instructions (Appendix A).

DNA preparations were quantified by measuring the OD at 260 nm and 280 nm. A ratio (260 nm/ 280 nm) was calculated and used to check the purity of preparations. Samples that did not fall into the range between 1.8 and 1.9 were excluded. The presence of DNA was assured by running 10 μl of each preparation on a 1.0 % agarose gel for 1.5 hrs at 80 Volts. DNA bands were detected by staining with ethidium bromide and visualised under ultra violet light on a GelDoc 2000 system (Bio-Rad, USA).

Optimisation of AP-PCR

Many variables can affect the reproducibility of AP-PCR (Tyler *et al,* 1997); therefore, several combinations of conditions and reagent concentrations were tested during the optimisation. The varying conditions included the annealing temperature, since one of the characteristics of the arbitrary primers is to amplify the DNA template at lower temperatures than the standard PCR (Williams *et al,* 1990). The optimisation of the PCR reagents included various concentrations of primer, DNA template, *Taq* polymerase, $MgCl_2$, dNTPs and buffer (Table 4.2). The initial AP-PCR was based on published AP-PCR methods for intestinal spirochaetes (Duhamel *et al,* 1995a; Dugourd *et al,* 1996). Thereafter, optimal conditions were developed empirically as has been recommended for AP-PCR (Towner and Grundmann, 2001).

Table 4.2. Concentrations of PCR reagents per reaction used for the optimisation of AP-PCR.

Reagent	Concentration				
$MgCl_2$	2 mM	3mM	4mM	5mM	
Taq	0.5 U	1 U	1.5 U	2 U	2.5 U
dNTPs	200 μM				
Primer	12.5 pM	25 pM	31.25 pM	62.5 pM	125 pM
DNA template	12.5 ng	25 ng	31.25 ng	62.5 ng	

DNA template

Optimisation was initially attempted with DNA extracted by the phenol chloroform method but due to the inconsistent results of the initial attempts the DNA from bacterial cells was extracted using a commercial kit. The concentrations of DNA template varied from 12.5 to 62.5 ng per reaction (12.5 ng/μl).

Arbitrary primers

The primers (Table 4.3) were purchased from Sigma-Genosys, UK. The primers were resuspended in 250 μl of molecular grade water (Sigma, UK), and prepared of a working stock concentration of 12.5 pM/μl. Different concentrations of primers were tested and varied from 12.5 to 125 pM (1 to 10 μl). The primers were used independently to screen the whole set of isolates of *B. pilosicoli*.

Table 4.3. Primer specifications.

Primer	Length (no. bases)	Sequence (5'-3')	G+C*	Tm**
AP002	10	ATGTATCTGC	40 %	23.3
AP1247	10	AAGAGCCCGT	60 %	37.1
AP1254	10	CCGCAGCCAA	70 %	47.2
AP1283	10	GCGATCCCCA	70 %	44.6
APKF	19	CACGCACACGCACAGAGAG	63 %	67.4
APKG	18	CACACGCACACGGAAGAA	55.5 %	64.9

* Guanine+Cytosine
** Melting temperature

$MgCl_2$
$MgCl_2$ influences the specificity and efficiency of the PCR. High concentrations increase the yielding of the PCR and low concentrations increase the specificity but decrease the yield. The working stock solution of $MgCl_2$ was 50 mM and the concentrations of $MgCl_2$ tested varied from 2 to 5 mM.

dNTPs
The concentration of each dNTP was 200 μM per AP-PCR assay reaction.

Taq polymerase
Concentration of DNA polymerase influences the performance of the PCR, low concentration may give poor yielding and high concentration may produce non-specific reaction. It has also been reported that the efficacy the *Taq* polymerase may vary from between manufacturers (Tyler *et al,* 1997). The concentrations tested varied from 0.5 to 2.5 units of *Taq* polymerase (Life Technologies- Gibco BRL, UK).

AP-PCR final concentrations
Optimised AP-PCR assays were performed using 3 mM $MgCl_2$, 200μM each dNTPs, 2 U *Taq* polymerase and 1X PCR buffer (Life Technologies- Gibco BRL, UK), 62.5 pM (5.0 μl) primer and 31.25 ng of DNA template (2.5 μl) in 50 μl final volume using molecular grade water (Sigma, UK). Negative controls (no DNA template added) were included in each assay.

Thermocycling programmes
Optimisation of thermocycling conditions included variation in the total number of cycles from 35 to 40, and time of the final extension cycle varying from 2 to 5 minutes. The thermocycling programmes were the same for all primers except for the annealing temperature (Table 4.4). All of the thermocycling reactions were carried out in an Express thermal cycler (Hybaid, UK).

Table 4.4. Thermocycling programmes.

1. Initial denaturing	2a. Denaturing	2b. Annealing	2c. Extension	3. Final extension
94°C 1 min- 1 cycle	94°C 1 min- 35 cycles	**AP002** - 38°C 1 min- 35cycles **AP1247**- 40°C 1 min- 35 cycles **AP1254**- 52°C 1 min- 35 cycles **AP1283**- 50°C 1 min- 35 cycles **APKF** - 53°C 1 min- 35 cycles **APKG** - 53°C 1 min- 35 cycles	72° C 1 min- 35 cycles	72° C 2 min- 1 cycle

Polyacrylamide gel electrophoresis (PAGE) and staining

The resulting PCR products were displayed on 12 % PAGEs (Appendix A) along with the ØX174/*Hae III* genetic marker (11 fragments ranging in size from 72 to 1353 bp; Sigma, UK) and stained with silver nitrate (Sigma, UK). Briefly, the gel was removed from the cassette and put in 100 ml fixer (50 ml ethanol, 2.5 ml acetic acid and 447.5 ml distilled water) shaking slowly for 10 minutes, then stained with silver nitrate at a concentration of (0.19 g/100 ml distilled water) for 10 minutes at slow rotation and washed twice with tap water. The stained gels were then developed for 10 minutes at slow rotation using 3.0 % NaOH (100 ml), and to catalyse the reaction 0.75 ml formaldehyde was added. The characteristic yellow colour of gels and black DNA fragments started to show between 5 and 10 minutes after the gel was in contact with developer solution. Once the desired colour intensity was achieved, the reaction was stopped by discarding the developer. The gel was then put in a stop solution for 10 min. at slow rotation. Stained gels were preserved in plastic bags and then scanned using the computer system GelDoc 2000 (Bio-Rad, USA) for further analysis.

Banding patterns and data analysis

Images of the resulting banding patterns were captured using Diversity Database fingerprinting software version 2.0 (Bio-Rad, USA). All bands generated by each primer were transformed to a binary format (1,0), where 1= band present and 0= band absent. Further, a data set including all the fragment data of the four primers was created and designated as pooled data. Data as (1,0) format was used to generate

distance matrices on RAPDDIST version 2.0 computer programme (Black and Antolin, 1997), based on Nei's genetic distance method, using a 1000 bootstrap value. The genetic distance is based on the identity of genes between populations, and is defined as D=-log$_e$I, where I is the normalised identity of genes between two populations. Nei's genetic distance method measures the accumulated nucleotide substitutions per locus (Nei, 1972). Phylogenetic trees were constructed by Neighbor-Joining method using the NEIGHBOR computer programme which builds phylogenetic trees by successive clustering of lineages, setting branch lengths as lineages join, then and a consensus tree was generated using the CONSENSE computer programme, both programmes were from PHYLIP version 3.5, Phylogenetic Inference Package (Felsenstein, 1993). Phylogenetic trees were visualised on the TREEVIEW version 1.5 computer programme (Page, 1998) as phylograms for each primer and as a cladogram (1000 bootstrap) for the pooled data set. Further, the pooled data set was also analysed by parsimony method on the PAUP version 4.0 computer programme, Phylogenetic Analysis Using Parsimony (Swofford, 1996), which states that the optimum tree is such that explains the data most efficiently by the fewest number of character changes (Farris, 1970). The genetic distance is measured as number of genetic changes that occurred from one individual to the other.

Bootstrap is defined as a statistical method based on repeated random sampling with replacement from an original sample to provide a collection of new pseudoreplicate samples.

Phylogram is a tree that depicts inferred historical relationships among entities, the branches are drawn proportional to the amount of inferred character change.

Cladogram is a tree that depicts inferred historical branching relationships among entities. Unless otherwise stated, the branch lengths in a cladogram are arbitrary; only the branching order is significant.

Results

The presence of DNA extracted from the set of isolates of *B. pilosicoli* was observed on 1.5 % agarose gel (Fig. 4.1).

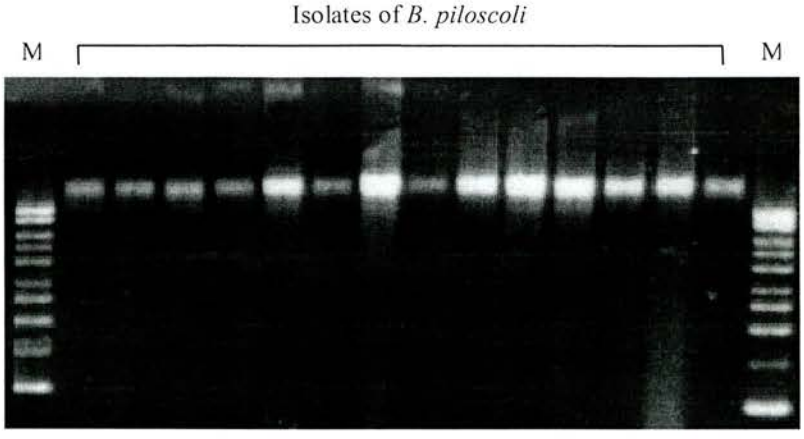

Fig. 4.1. Example of an agarose gel (1.5 %) displaying the DNA extracted from *B. pilosicoli* isolates. M, DNA ladder 1kb (0.5-10 kb)

The reproducibility of the AP-PCR assays was obtained after several assays using DNA template from strain ATCC-51139 and the isolates P4 and P6 cultured at different times to prove reproducibility. The assays for the whole set of isolates were repeated at least twice to confirm repeatability of the banding patterns generated by each primer.

Each of the four primers used in this study generated similar number of DNA fragments from *B. pilosicoli* reference strain ATCC-51139 ranging from 12 to 16. The total number of bands generated from the whole set of field isolates ranged from 37 to 45 (Table 4.5). The total number of fragments generated by all primers gave a pooled data set with a total of 163 DNA fragments (i.e. 37 + 45 + 37 + 44 = 163). The primers AP1283 and APKF did not produce satisfactory results irrespective of conditions used so, these primers were not included in the banding pattern analysis.

The banding pattern generated from *B. hyodysenteriae* by each primer was distinguishable from the respective banding patterns of *B. pilosicoli* (Fig. 4.2a and 4.2b, DNA banding patterns generated by AP-002 given as an example). The negative controls confirmed that no non-specific amplification occurred using the optimised AP-PCR.

Table 4.5. Bands generated from type strain ATCC-51139 and field isolates of *B. pilosicoli* by each primer.

Primer	Bands generated from ATCC-51139	Total number of bands generated from the whole set of field isolates	Size range of generated bands
AP002	13	37	2034-277 bp
AP1247	16	45	2441-290 bp
AP1254	12	37	2417-372 bp
APKG	16	44	2341-369 bp

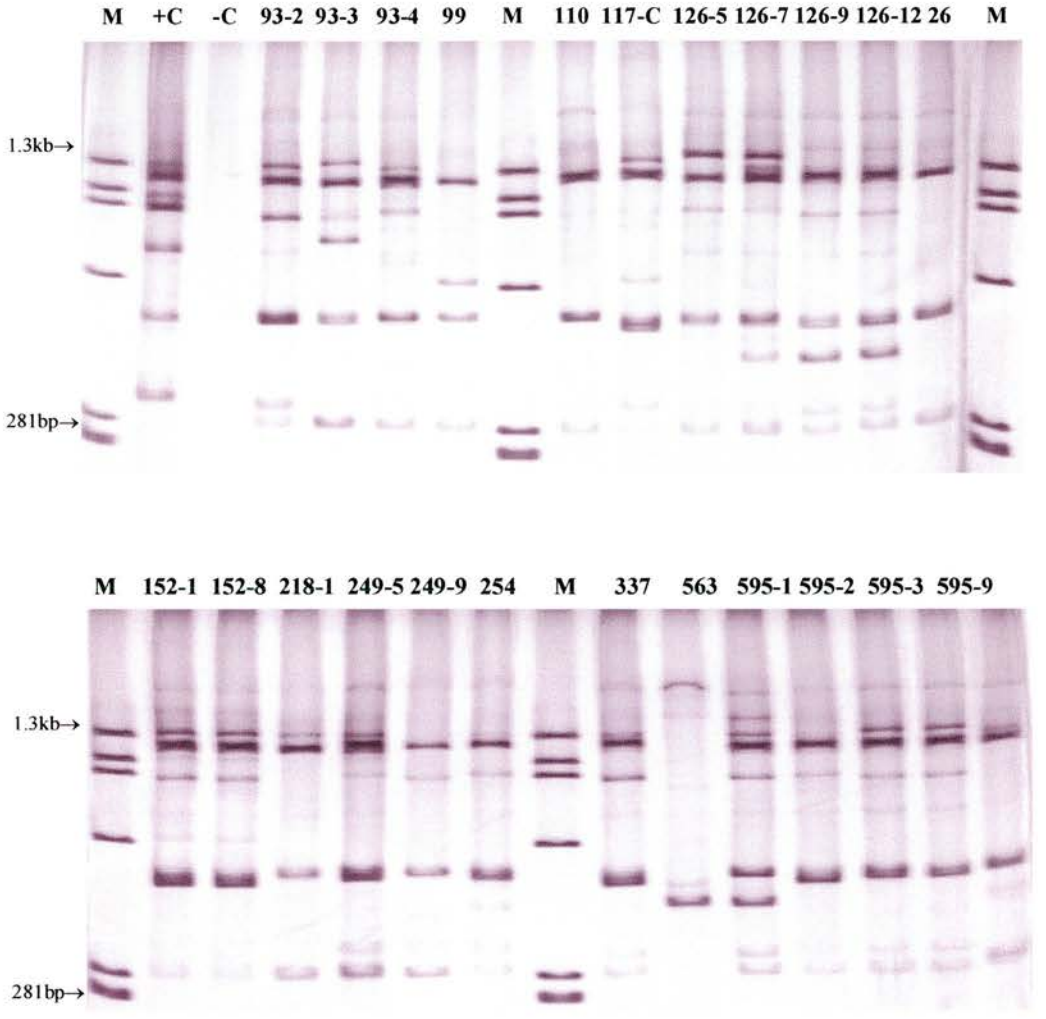

Fig. 4.2a. DNA banding patterns generated by AP-002 from *B. pilosicoli* isolates. M, molecular marker; +C, positive control; -C, negative control

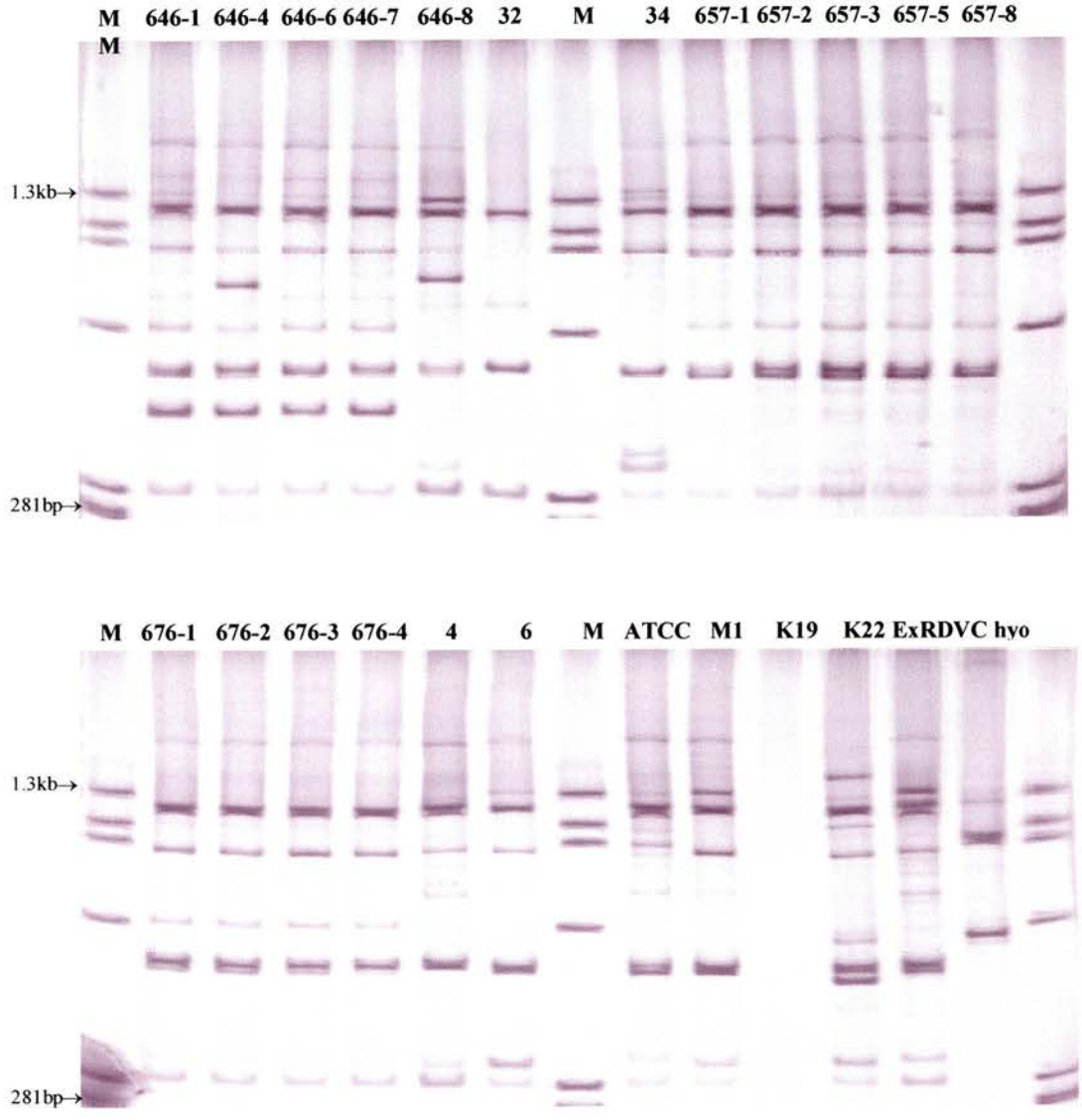

Fig. 4.2b. DNA banding patterns generated by AP-002 from *B. pilosicoli* isolates. M, molecular marker.

Characterisation of isolates with AP-002

The most common band generated by this primer was the band type 1 of size 2034 bp found in 91.4 % (44) of the isolates, followed in frequency by band type 35 of 306 bp found in 78.7 % (38) of the isolates, band type 10 of 1199 bp in 76.6 % (37) of the isolates, band type 8 of 1344 bp in 72.3 % (35) of the isolates, and band type 25 of 510 bp in 68.0 % (33) of the isolates. Four of the most frequent band types generated by this primer were present on the reference strain ATCC-51139. Details of band frequencies are shown in Appendix C. The DNA template of the dog isolate K19 was not amplified by this primer.

Three main groups were observed on the phylogram of this primer: group 1 contained the outgroup *B. hyodysenteriae*, group 2 was represented by 2 of the single isolates, P26 and P277, and group 3 included 44 isolates (Fig. 4.3). Group 3 was subdivided into 9 clusters, of which cluster 1 consisted of two single isolates P32 and P99 pairing together. Cluster 2 paired together two of the single isolates, P110 and P563. Cluster 3 consisted of only one isolate, the isolate P249-9 one of the two isolates from farm P249. Cluster 4 comprised 5 isolates, including all the isolates from farm P126 together with the isolate P254. Cluster 5 included 3 isolates, of which each isolate was a multiple isolate (P249-5, P646-8 and P93-4) of different farms. Cluster 6 had 4 isolates, including the multiple isolates P93-2 and P595-9, the single isolate P34, and the reference strain ATCC-51139. Cluster 7 included two of the multiple isolates from farm P595. Cluster 8 was the second biggest cluster with 11 isolates including 4 of the 5 multiple isolates from farm P646 and all the multiple isolates from farm P657, the multiple isolate P93-3, and the human isolate ExRDVC was also included in this cluster. Cluster 9 was the biggest cluster with 14 isolates including all the multiple isolates from farm P676, the two multiple isolates from farm P152, one of the multiple isolates from farm P595, the single isolates P4, P337, P218-2, P6, and the UK reference strain M1, and the dog isolate K22 which paired together with single isolate P117-C.

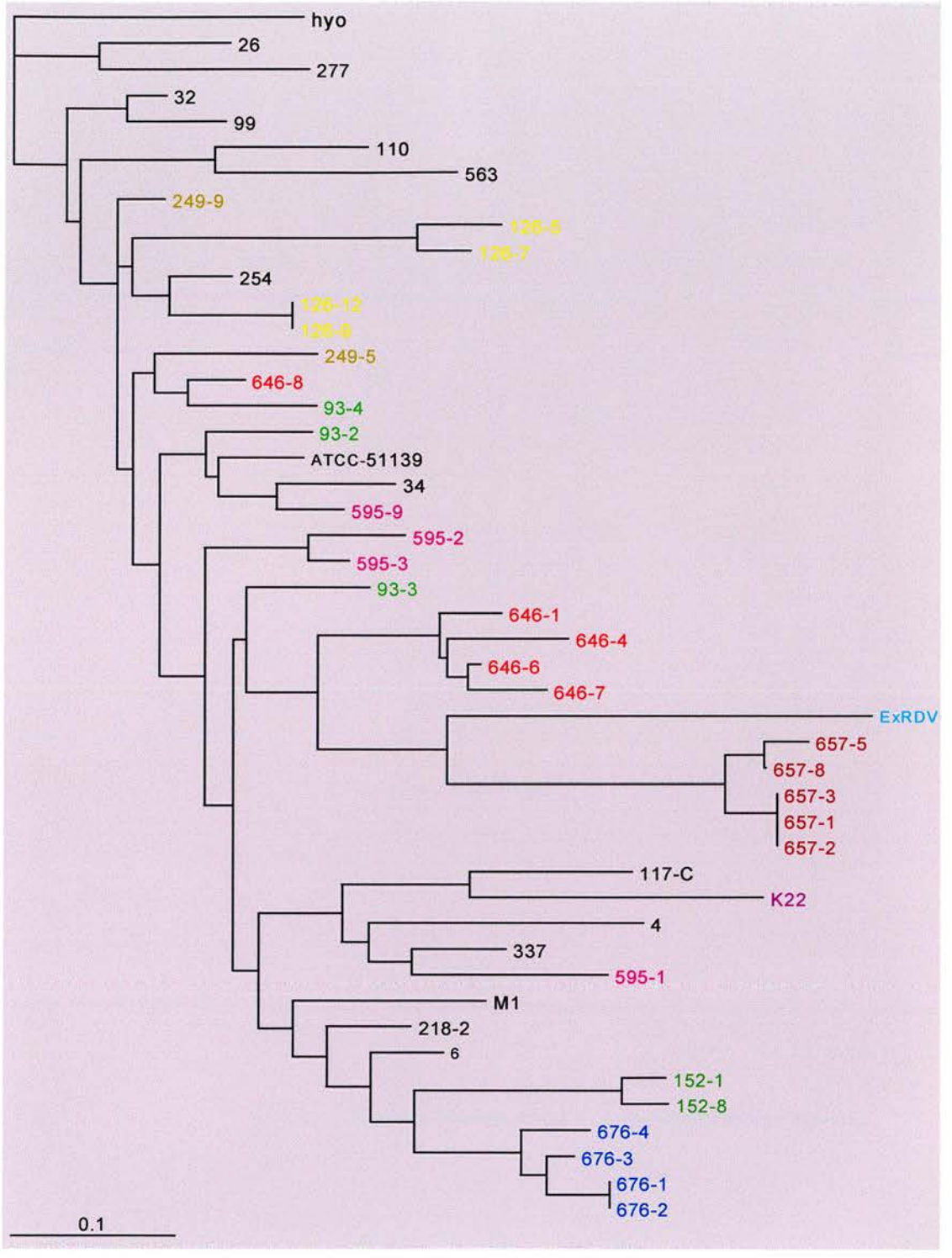

Fig. 4.3. Phylogram generated by AP-002 showing genetic distances between *B. pilosicoli* isolates.

Continuing of Fig, 4.3, genetic distances generated by AP-002 corresponding to the length of branches on phylogram. Branches/clusters are delimited by parenthesis and genetic distances between clusters or individuals are given after a colon.

((((((**26**:0.08057,**277**:0.12913):0.05242,**hyo**:0.17808):0.03274,
(**32**:0.02416,**99**:0.06044):0.03691):0.00862,(**110**:0.09466,**563**:0.14894):0.08181):0
.02256,
249-9:0.02890):0.04515,((**254**:0.03847,(126-12:0.00000,126-
9:0.00000):0.07593):0.02256,
(126-5:0.05164,126-7:0.03296):0.17484):0.00901,(((((((**4**:0.16717,
(**337**:0.05739,595-1:0.11951):0.02563):0.01691,(**117-C**:0.09915,
K22:0.17955):0.07788):0.05128,(((**6**:0.04393,((152-1:0.02710,
152-8:0.02850):0.12680,(((676-1:0.00000,676-2:0.00000):0.03844,
676-3:0.01716):0.01519,676-4:0.04201):0.06557):0.02637):0.02717,
218-2:0.05131):0.02085,**M1**:0.11807):0.02110):0.01590,(((646-1:0.03777,
(646-4:0.07383,(646-6:0.00765,646-7:0.04795):0.01277):0.00473):0.07435,
((((657-1:0.00000,657-2:0.00000):0.00000,657-3:0.00000):0.03206,
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8:0.00059):0.02434):0.16985,ExRDVC:0.25988):0.07916):0.04403,
93-3:0.07562):0.00818):0.01754,(595-2:0.05935,595-
3:0.02525):0.06357):0.02746,
(((**34**:0.07292,595-9:0.04148):0.03589,**ATCC**:0.05256):0.00745,
93-2:0.06537):0.02807):0.01662,(249-5:0.09964,(646-8:0.03520,

Characterisation of isolates with AP-1247

The most common bands generated by this primer were the band type 23 of 795 bp found in 66.6 % (32) of the isolates and band type 20 of 905 bp found in 45.8 % (23) of the isolates. Three band types were found in 43.8 % (21) of the isolates, they were band type 17 of 1149 bp, band type 35 of 504 bp and band type 39 of 416 bp. The band type 28 of 661 bp was found in 39.5 % (19) of the isolates. Five band types were found in 22.9 % (11) of the isolates i.e. band type 5 of 1808 bp, band type 7 of 1662 bp, band type 12 of 1362 bp, band type 15 of 1260 bp, band type 18 of 1058 bp and band type 42 of 354 bp. Eight of the most common band types generated by this primer were present on the reference strain ATCC-51139. Band frequencies are shown in Appendix C.

The phylogram of this primer separated the isolates into 3 main groups (Fig. 4.4): group 1 the outgroup *B. hyodysenteriae;* group 2 consisted of 4 isolates, the single isolate P277 (also included in group 2 on the phylogram of AP002) paired together with the human isolate ExRDVC and two of the multiple isolates from farm P595 (isolates 3 and 9), and Group 3 included 43 isolates (Fig. 4.4). Group 3 was subdivided into 4 clusters, of which cluster 1 consisted of 13 isolates including 3 of the 5 multiple isolates from farm P126, all the multiple isolates from farm P93, one of the multiple isolates from farm P249 and the single isolates P117-C, P563, P254, P99, P110 and P337. The single isolate P254 also clustered together with the multiple isolates from farm P126 on the phylogram generated with AP002. Cluster 2 consisted of 8 isolates: the two multiple isolates from farm P152, two of the four multiple isolates from farm P595, one of the multiple isolate P249-5, the dog isolate K19, and the single isolates P218-2 and P26. The two multiple isolates from farm P152 were also found clustering together with the reference strain M1 and the dog isolate K22 on the phylogram of AP002. Cluster 3 included 10 isolates: all the isolates from farm P676 together with the single isolate P4, the reference strain M1, the dog isolate K22, the reference strain ATCC-51139, and one of the multiple isolates from farms P657 and P126 (P657-1 and P126-9, respectively). Multiple isolates from farm P676 clustered together with the dog isolate K22

and the reference M1 on the phylogram of AP002. Cluster 4 was the biggest cluster comprising 12 isolates: all the multiple isolates from farm P646 and four of the multiple isolates from farm P657, together with the single isolates P6, P32 and P6. Isolates from farms P646 and P657 were also found forming a cluster on the phylogram of AP002.

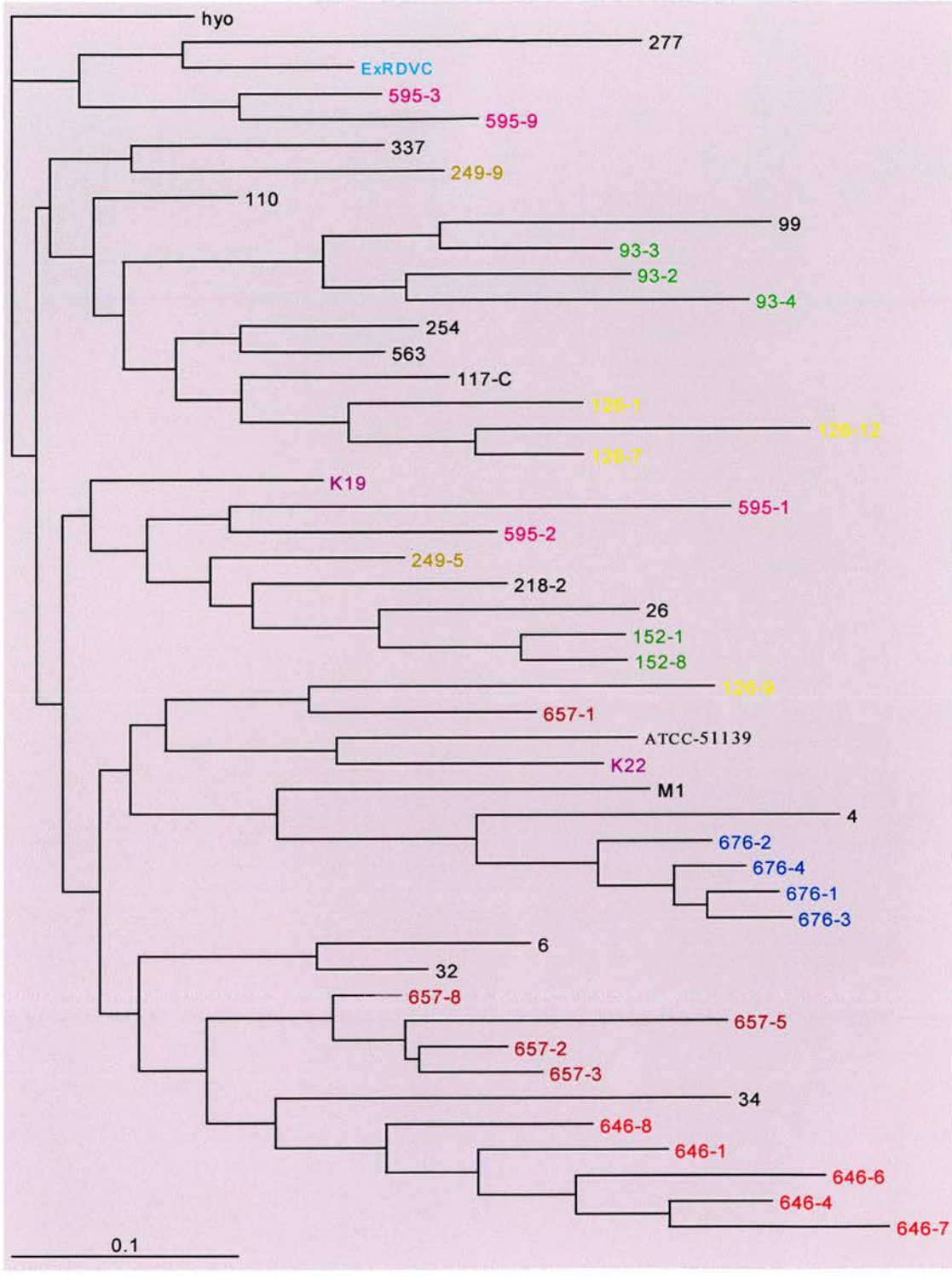

Fig. 4.4. Phylogram generated by AP1247 showing genetic distances between multiple isolates of *B. pilosicoli*.

Continuing of Fig 4.4, genetic distances generated by AP-1247 corresponding to the length of branches on phylogram. ***Branches/clusters are delimited by parenthesis and genetic distances between clusters or individuals are given after a colon.***

(((((**99**:0.14699,93-3:0.07611):0.05186,(93-2:0.09928,93-4:0.15202):0.03696):0.08890,((**254**:0.07900,**563**:0.06410):0.02849,(**117-C**:0.09210,(126-5:0.10370,(126-12:0.14802,126-7:0.04768):0.05635):0.04753):0.02907):0.02341):0.01300,110:0.06362):0.01947,(**337**:0.11228,249-9:0.13902):0.03606,((((((**4**:0.16101,(((676-1:0.03178,676-3:0.03722):0.01474,676-4:0.03181):0.03365,676-2:0.05042):0.05391):0.08849,**M1**:0.16524):0.06519,((126-9:0.17981,657-1:0.10049):0.06389,(**ATCC**:0.13329,K22:0.11801):0.07609):0.01570):0.01341,((6:0.09429,**32**:0.04881):0.07936,((**34**:0.20146,((646-1:0.08415,((646-4:0.04580,646-7:0.09730):0.04155,646-6:0.11040):0.04315):0.04072,646-8:0.09126):0.04893):0.03065,(((657-2:0.03872,657-3:0.05438):0.00634,657-5:0.14281):0.03213,657-8:0.03032):0.05591):0.03031):0.01711):0.01639,(((((**26**:0.11529,(152-1:0.04612,152-8:0.04698):0.06286):0.05606,**218-2**:0.11254):0.01908,249-5:0.08615):0.02818,(595-1:0.22244,595-2:0.11846):0.03680):0.02486,K19:0.10307):0.01234):0.01144,(((277:0.20413,ExRDVC:0.07617):0.04579,(595-3:0.06313,595-9:0.10597):0.07116):0.03003,**hyo**:0.08099):0.01102):0.00596);

Characterisation of isolates with AP-1254

The most frequent band type generated by this primer was the band type 34 of 436 bp found in 87.5 % (42) of the isolates followed by band type 17 of 848 bp in 85.4 % (41) of the isolates, band type 22 of 714 bp in 81.3 % (39) of the isolates, band type 5 of 1808 bp in 60.4 % (29) of the isolates, and band type 8 of 1573 bp in 54.2 % (26) of the isolates. Four of the most common band types generated by this primer were found on the reference strain ATCC-51139. Band frequencies are shown in Appendix C.

This primer separated the isolates into 3 main groups, group 1 being the outgroup *B. hyodysenteriae*, group 2 including only the single isolate P337 and group 3 including 46 isolates (Fig. 4.5). Group 3 was subdivided into 4 clusters, of which cluster 1 included 11 isolates, all the multiple isolates from farm P676, the two multiple isolates from farm P152, one of the multiple isolates from farm P249, the single isolates P218-2, P6, P4 and reference strain M1. The two multiple isolates from farm P152, and the single isolates P218-2, P6, P4, and the reference strain M1 also formed a cluster with multiple isolates from farm P676 on the phylogram of AP002, and the same applied to the single isolate P4 and reference strain M1 on the phylogram of AP1247. Cluster 2 included 9 isolates: all the multiple isolates from farm P595, the single isolates P277, P26, P254, the human isolate ExRDVC and one of the multiple isolates from farm P93. From these, the human isolate ExRDVC was also found forming a cluster together with multiple isolates from farm P595 and the single isolate P277 on the phylogram of AP1247. Cluster 3 consisted of 7 isolates of which one was the reference strain ATCC-51139 which clustered together with 2 of the multiple isolates from farm P126, one of the multiple isolates from farm P93 and the single isolate P99. The multiple isolates from farms P93, P126 and the single isolate P99 were also found clustering together on the phylogram of AP1247. Cluster 4 was the biggest cluster with 19 isolates including all the multiple isolates from farms P646 and P657, two of the multiple isolates from farm P126, one of the multiple isolates from farm P93, the single isolates P32, P563, P34, P99, and the two dog isolates K19 and K22. The isolates from farms

P646 and P657 were also found clustering together on phylograms of AP002 and AP1247; moreover, the dog isolate K22 was also found clustering together with these isolates on the phylograms of AP002 and AP1247.

The single isolates P277 and P26 were also found pairing together on the phylogram of AP002.

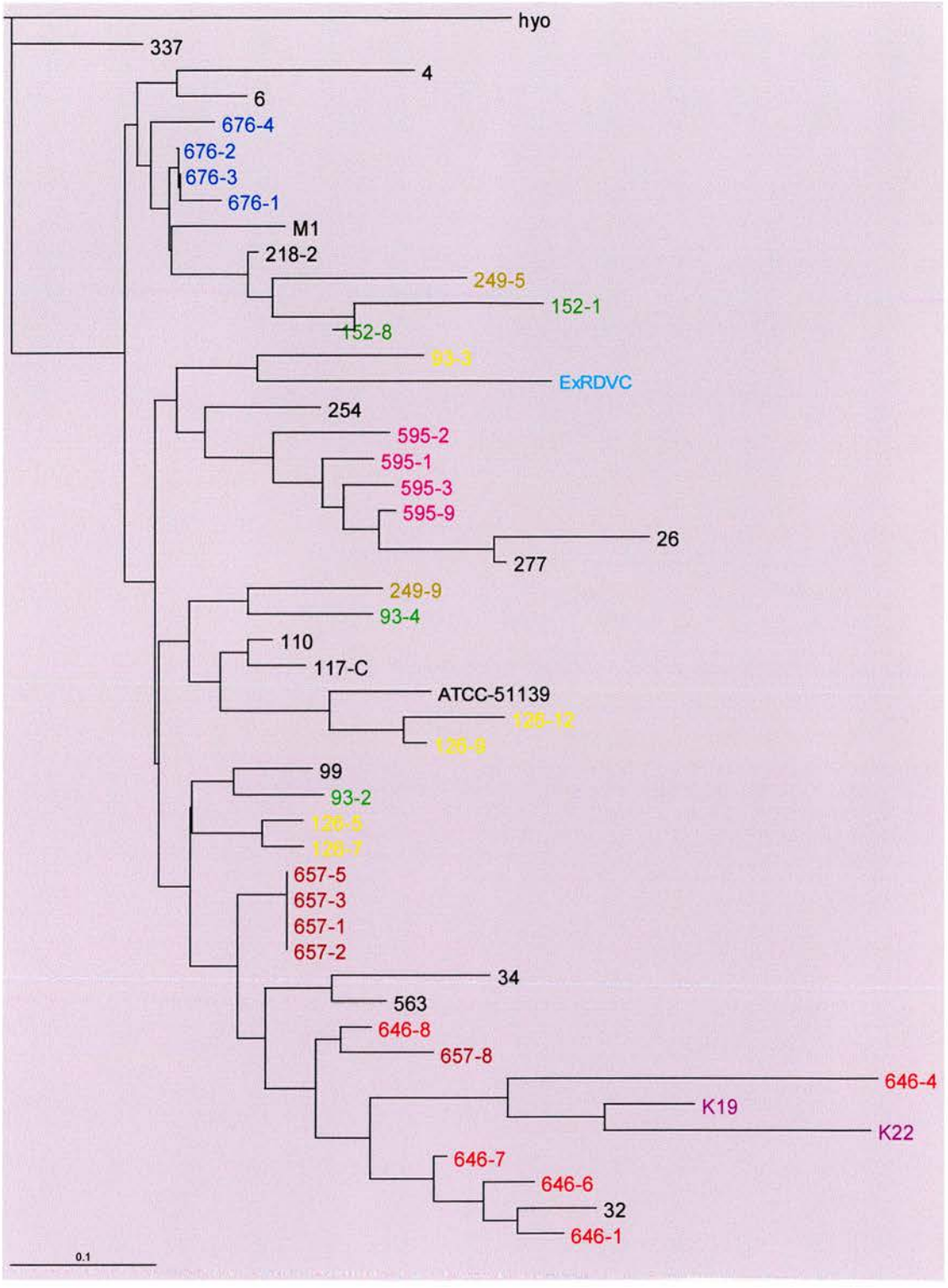

Fig. 4.5. Phylogram generated by AP1254 showing genetic distances between multiple isolates of *B. pilosicoli*.

Continuing of Fig. 4.5, genetic distances generated by AP1254 corresponding to the length of branches on phylogram. Branches/clusters are delimited by parenthesis and genetic distances between clusters or individuals are given after a colon.

(((((**99**:0.14699,93-3:0.07611):0.05186,(93-2:0.09928,93-4:0.15202):0.03696):0.08890,
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646-8:0.09126):0.04893):0.03065,(((657-2:0.03872,657-3:0.05438):0.00634,
657-5:0.14281):0.03213,657-8:0.03032):0.05591):0.03031):0.01711):0.01639,
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218-2:0.11254):0.01908,249-5:0.08615):0.02818,(595-1:0.22244,
595-2:0.11846):0.03680):0.02486,K19:0.10307):0.01234):0.01144,
(((277:0.20413,ExRDVC:0.07617):0.04579,(595-3:0.06313,595-9:0.10597):0.07116):0.03003,

Characterisation of isolates with AP-KG

The most common band type generated by this primer was the band type 28 of 672 bp found in 93.7 % (45) of the isolates, followed by band type 29 of 646 bp in 89.5 % (43) of the isolates, band type 27 of 689 bp in 75.0 % (36) of the isolates, band type 30 of 620 bp in 72.9 % (35) of the isolates, and band type 21 of 846 bp in 68.7 % (33) of the isolates. Four of the most common band types generated by this primer were present in the reference strain ATCC-51139. Band frequencies are shown in Appendix C.

This primer separated the isolates again in 3 groups. Group 1 consisted of the outgroup *B. hyodysenteriae*; group 2 comprised two of the multiple isolates from farm P595 and group 3 included 45 isolates, and group 3 was subdivided in 6 clusters of which cluster 1 had only one isolate, the single isolate P218-2 (Fig 4.6). Cluster 2 included two isolates, the human isolate ExRDVC and the dog isolate K22 pairing together. Cluster 3 included only the reference strain ATCC-51139. Cluster 4 included four isolates, one of the multiple isolates from farm P657, one of the multiple isolates from farm P249, the single isolate P34 and the dog isolate K19. Cluster 5 was the second biggest cluster with 18 isolates including all the multiple isolates from farms P676 and P93, both of the multiple isolates from farm P152, the single isolates P4, P10, P6, P99, P26, P32, P277, P563 and the reference strain M1. The multiple isolates from farm P676 were also seen forming a cluster together with the reference strain M1 and the single isolate P6 on the phylograms of AP002, AP1247 and AP1254, and also with the two multiple isolates from farm P152 on the phylograms of AP002 and AP1254. Cluster 4 was the biggest cluster with 19 isolates including all the multiple isolates from farms P646 and P126, four of the five multiple isolates from farm P657, two of the multiple isolates from farm P595, one of the multiple isolates from farm P249 and the single isolates P337, P254, P117-C. The isolates from farms P646 and P657 were also found forming a cluster together on the phylograms of AP002, AP1247 and AP1254. Also multiple isolates from farm P126 were found forming a cluster with the multiple isolates P249-9 and single isolate P254 on the phylogram of AP002, and with the single isolates P254 and

P117-C on the phylogram of AP1247, and with the multiple isolate P249-9 and single isolate P117-C on the phylogram of AP1254. Another of the main clusters of group 3 included the dog isolate K19 which paired together with the multiple isolate P249-5. The human isolate ExRDVC paired together with the other dog isolate K22.

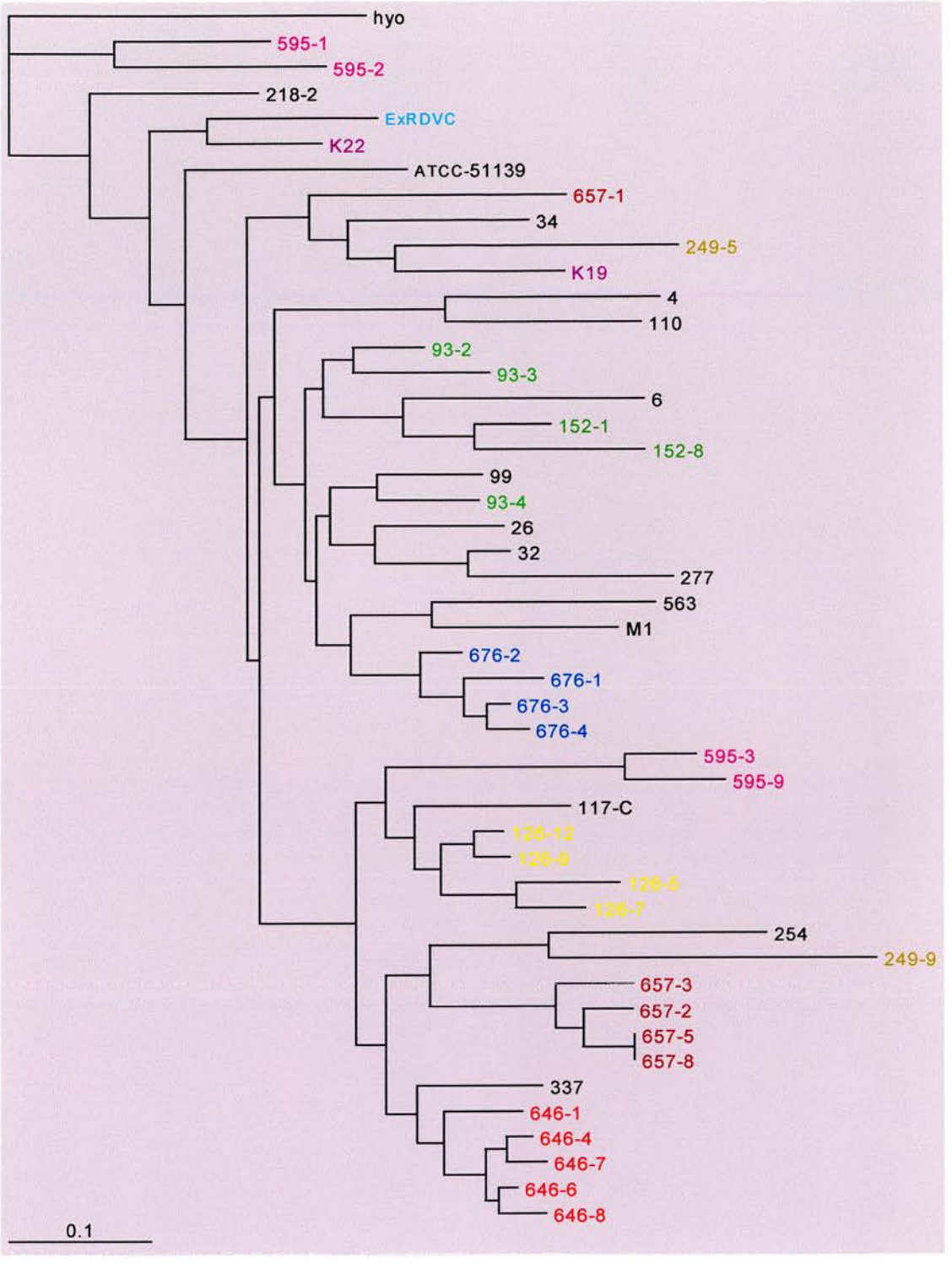

Fig. 4.6. Phylogram generated by APKG showing genetic distances between multiple isolates of *B. pilosicoli*.

Continuing of Fig. 4.6, genetic distances generated by APKG corresponding to the length of branches on phylogram. Branches/clusters are delimited by parenthesis and genetic distances between clusters or individuals are given after a colon.

(((**26**:0.09091,(**32**:0.02962,**277**:0.14368):0.06574):0.03182,
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((((**34**:0.12779,(249-5:0.19901,K19:0.11949):0.03336):0.02689,
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595-2:0.14859):0.07451,**hyo**:0.25114):0.05715):0.04194,(ExRDVC:0.11991,
K22:0.08079):0.04056):0.02509,**ATCC**:0.15602):0.04302):0.00880,
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657-8:0.00000):0.03608):0.01926,657-3:0.05404):0.08857):0.03097,
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((**117-C**:0.10959,((126-12:0.02096,126-9:0.02554):0.02341,
(126-5:0.07221,126-7:0.04839):0.05334):0.01856):0.01982,
(595-3:0.05003,595-9:0.07057):0.16783):0.02126):0.06735):0.01037):0.02178,
((**6**:0.16916,(152-1:0.05389,152-8:0.11941):0.05009):0.05647,
(93-2:0.05036,93-3:0.09624):0.02142):0.01239):0.00742);

Individual primers showed some consistency of cluster forming of the multiple isolates but patterns of relatedness generated by each primer had degrees of variation. Data generated by each primer were pooled (four primers = 163 fragments) to give more consistency to the analysis of the cluster forming and better definition to the genetic relatedness of isolates.

The genetic distances were calculated using two methods: distance method (Nei, 1972) which measures the accumulated allelic differences per locus and parsimony method (Farris, 1970) which explains genetic variation by the number of evolutionary steps or transformations from one character state to another. Thus, the reference strain ATCC-51139 had a genetic distance to the isolate of *B. hyodysenteriae* at the level of [(distance method) d=0.456 and (parsimony) d=92]. The human isolate ExRDVC was more distantly related to the reference strain ATCC-51139 [(distance method) d=0.418 and (parsimony) d=79] than the reference strain ATCC-51139 to the dog isolates K19 [(distance method) d=0.329 and (parsimony) d=51] and K22 [(distance method) d=0.408 and (parsimony) d=50]. The reference strain M1 had a genetic distance to *B. hyodysenteriae* at the level of [(distance method) d=0.444 and (parsimony) d=120], to the human isolate ExRDVC [(distance method) d=0.406 and (parsimony) d=107], and to the dog isolates K19 [(distance method) d=0.371 and (parsimony) d=106] and K22 [(distance method) d=0.450 and (parsimony) d=105] (Table 4.6).
Interestingly, the most closely related isolate to the reference strain ATCC-51139 was the dog isolate K19 (distance method d=0.329 and parsimony d=51) and the most genetically distant was as expected, the isolate of *B. hyodysenteriae* (distance method d=0.456 and parsimony d=92). More interesting, the dog isolate K19 was more closely related to the reference strain ATCC-51139 than the reference strain M1 to the reference strain ATCC-51139 (distance method d=0.366 and parsimony d=76). Conversely, the most closely related isolate to the UK reference strain M1 was the multiple isolate P676/2 and the most distantly related was again, as expected, the isolate of *B. hyodysenteriae* (distance method d=0.444 and parsimony d=120) (Table 4.6).

Table 4.6. Genetic comparisons between isolates of *B. pilosicoli* from different hosts and to *B. hyodysenteriae* by distance method and *parsimony*.

		Distance method					
Parsimony		**ATCC-51139**	**M1**	***B. hyodysenteriae***	**K19 (dog isolate)**	**K22 (dog isolate)**	**ExRDVC (human isolate)**
	ATCC-51139		0.366	0.456	0.329	0.408	0.418
	M1	*76*		0.444	0.371	0.450	0.406
	B. hyodysenteriae	*92*	*120*		0.461	0.540	0.448
	K19 (dog isolate)	*51*	*106*	*111*		0.305	0.423
	K22 (dog isolate)	*50*	*105*	*110*	*31*		0.502
	ExRDVC (hum. isolate)	*79*	*107*	*81*	*98*	*97*	

Pooled data (phylogram)

The phylograms by distance method and parsimony generated from pooled data gave clearer definition of the cluster forming and more accurate values of the genetic distances than those detected by each primer (Figs. 4.7 and 4.8).

On the phylogram of pooled data produced by the distance method it was observed that within the multiple isolates some sets were more genetically heterogeneous than others. The two multiple isolates from farm P152 were highly similar showing a genetic distance at the level (d= 0.109), whereas the two multiple isolates from farm P249 were more distant (d= 0.433). Within the three multiple isolates from farm P93, the isolates P93-2 and P93-4 were more closely related (d=0.252) than were P93-2 and P93-3 (d=0.264), or P93-3 and P93-4 (d=0.307). Among the multiple isolates from farm P126, the isolates P126-5 and P126-7 were more closely related (d= 0.116) than the isolates P126-9 and P126-12 (d= 0.123); these two pairs of isolates were genetically separated from each other by (d= 0.121). Within the multiple isolates from farm P595, the isolates P595-3 and P595-9 were slightly more closely related (d= 0.129) than the isolates

P595-1 and P595-2 (d= 0.272), these two pairs of isolates were separated by genetic distance at the level of (d=0.138). Of the four multiple isolates from farm P676, the isolates P676-1 and P676-3 were more closely related (d= 0.056) than the other two isolates P676-2 and P676-4 were from them (d= 0.107 and d= 0.115, respectively). As for farm P646, the most closely related isolates of this set were the isolates P646-1 and P646-6 (d= 0.109) compared to the isolates P646-4 and P646-7 (d=0.152), and the most distantly related from those two pairs was the isolate P646-8 (d= 0.239 and d= 0.295, respectively). Of the five isolates from farm P657, the pair of isolates P657-2 and P657-3 were more closely related (d= 0.043) than the other three isolates P657-5, P5657-8 and P657-1 to that pair (d= 0.120, d= 0.106 and d= 0.237, respectively) (Fig. 4.7).

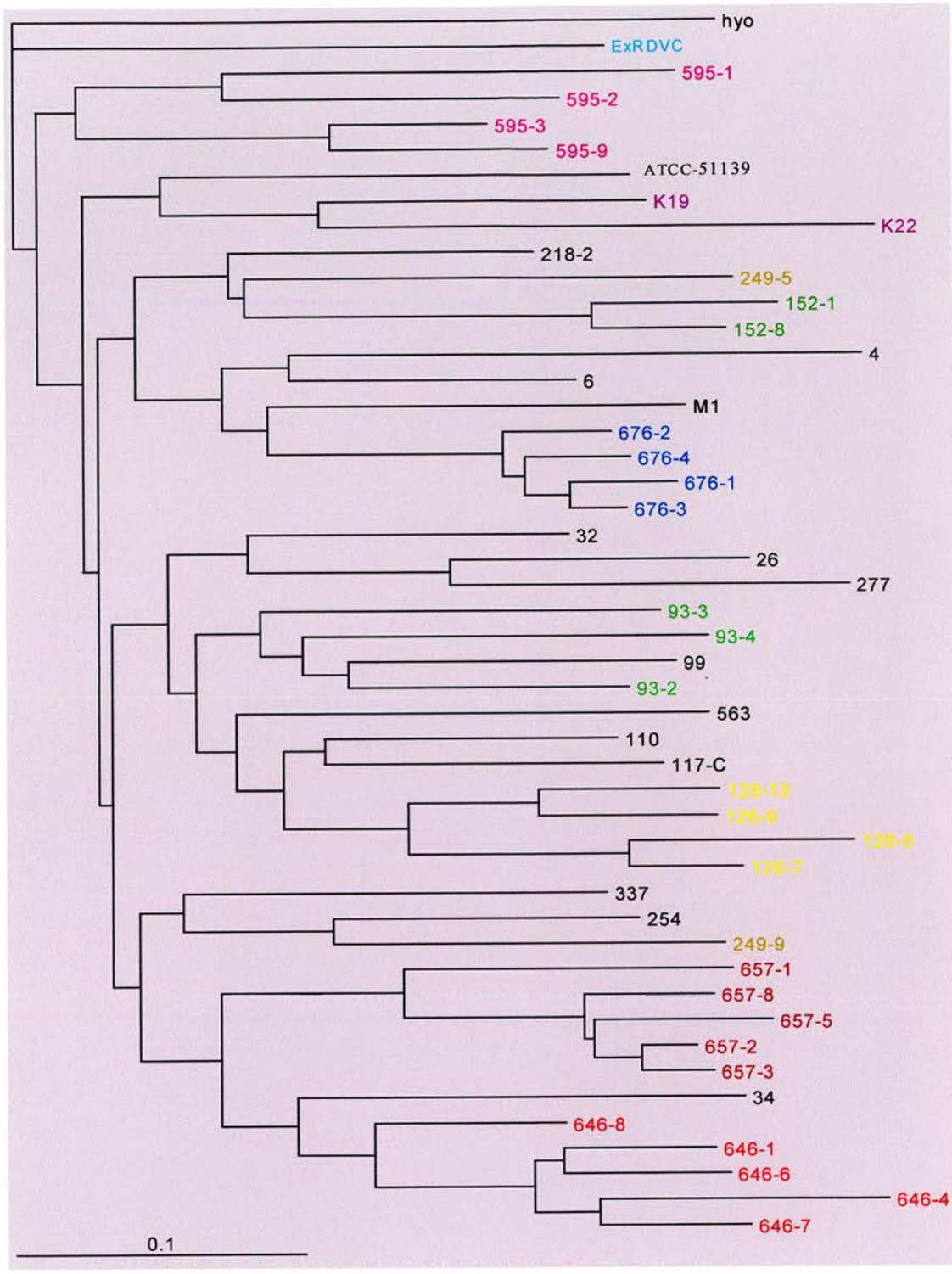

Fig. 4.7. Phylogram of pooled data (163 DNA fragments) generated by distance method showing genetic distances between multiple isolates of *B. pilosicoli*.

Continuing of Fig. 4.7, genetic distances generated by distance method from pooled data (four primers) corresponding to the length of branches on phylogram. Branches/clusters are delimited by parenthesis and genetic distances between clusters or individuals are given after a colon.

(((((**26**:0.10333,277:0.13837):0.07007**,32**:0.11123):0.02749,
((((**99**:0.11348,93-2:0.09742):0.01572,93-4:0.14038):0.01455,
93-3:0.13855):0.02247,(((**110**:0.10134**,117-C**:0.11726):0.01433,
((126-12:0.06244,126-9:0.06146):0.04529,(126-5:0.07794,126-
7:0.03906):0.07644):0.04323):0.01618,
563:0.16356):0.01404):0.00933):0.01964,(((**34**:0.15446,(((646-1:0.05257,
646-6:0.05753):0.00989,(646-4:0.10005,**646-7**:0.05205):0.02239):0.05566,
646-8:0.06628):0.02645):0.02629,(657-1:0.11371,(((657-2:0.01913,
657-3:0.02477):0.01640,657-5:0.06160):0.00345,657-
8:0.04488):0.06249):0.06315):0.02800,
((**254**:0.10613,249-9:0.13557):0.05147,337:0.14653):0.01517):0.00944):0.00461,
((((595-1:0.15686,595-2:0.11664):0.05101,(595-3:0.05498,
595-
9:0.07592):0.08789):0.01403,(ExRDVC:0.20564,**hyo**:0.24376):0.00810):0.01608
,
(**ATCC**:0.16292,(K19:0.11366,K22:0.19264):0.05478):0.02709):0.00511,(((**4**:0.1
9851,
6:0.09949):0.02289,((((676-1:0.03708,676-3:0.01972):0.01531,
676-4:0.03634):0.00751,676-
2:0.03727):0.08174,**M1**:0.14440):0.01553):0.03030,

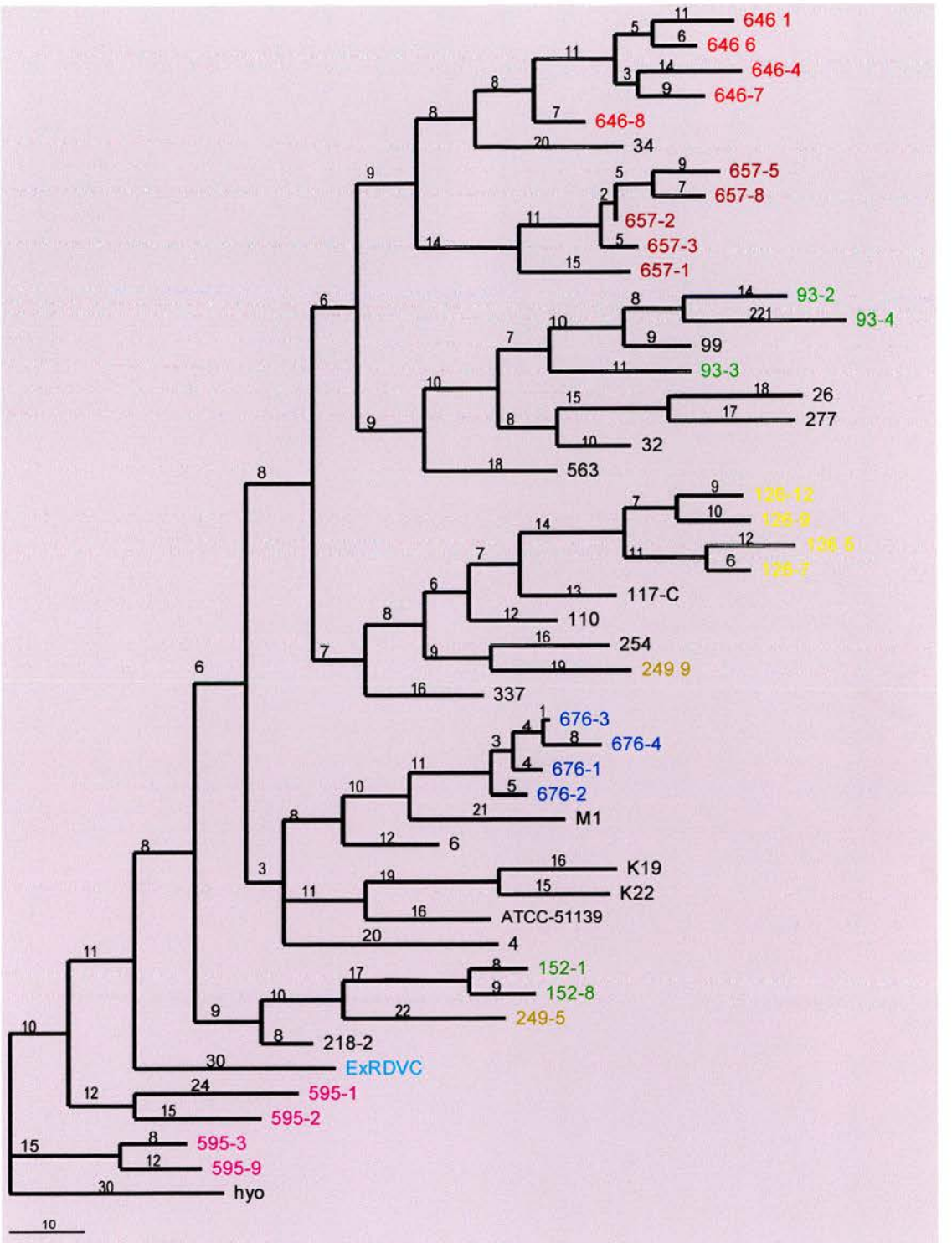

Fig. 4.8. Phylogram of pooled data (163 DNA fragments) generated by parsimony method showing genetic distances between multiple isolates of *B. pilosicoli*

Overall, the mean genetic distance of the multiple isolates by the distance method ranked the multiple isolates from farm P676 as the most closely related. Those isolates had a mean genetic distance at the level of (d= 0.032) followed by the isolates from farm P657 (d=0.052), whereas the most distantly related were the isolates from farm P249 (d=0.152) (Table 4.7 and Fig. 4.7). The same results for the ranking of farms by the mean genetic distance were obtained with parsimony method in which the multiple isolates from farm P676 had a mean genetic distance at the level of (d=4.5), followed by the isolates from farm P657 (d=7.2). The most distantly related were the isolates from farm P249 (d=20.5) (Table 4.7 and Fig. 4.8).

By distance method, the mean genetic distance within isolates from farm P249 was higher (d=0.152) than the mean genetic distance between all farms (d=0.127). The dog isolates K19 and K22 had the same value of the mean genetic distance (d=0.152) as the one between the isolates from farm P249. This indicates that the genetic distance between multiple isolates from the same farm may be high since the dog isolates were not epidemiologically related.

By parsimony method the mean genetic distances between the multiple isolates from three of the farms i.e. P595 (d=14.75), P93 (d=18.33) and P249 (d=20.5) were higher than the mean genetic distance of the isolates (unrelated) between farms (d=14.53). The mean genetic distance of the multiple isolates from farms P93 and P249 were higher (d=18.33 and d=20.5, respectively) than that of the two unrelated dog isolates (d=15.5) (Table 4.7). This suggested that the genetic variation of multiple isolates of *B. pilosicoli* could be very high since multiple isolates of the same farm showed higher genetic distance than that of unrelated isolates.

Table 4.7. Mean genetic distance of single and multiple isolates of *B. pilosicoli*.

Farm (no. isolates per farm)	Mean genetic distance	
	Distance method	Parsimony method
P676 (4)	0.032	4.50
P657 (5)	0.052	7.20
P152 (2)	0.056	8.50
P126 (4)	0.060	9.25
P646 (5)	0.065	9.40
P595 (4)	0.100	14.75
P93 (3)	0.125	18.33
P249 (2)	0.152	20.50
K19- K22	0.152	15.50
13 farms (13 single isolates)	0.127	14.53

Pooled data (cladogram)

The cladogram with 1000 bootstrap value generated from pooled data clearly defined the cluster forming of the multiple isolates from each farm according to their genetic relatedness; in other words, multiple isolates from the same farm tended to cluster together in most cases (Fig. 4.9). Some of the multiple isolates showed very high bootstrap values on the cluster forming, for instance, the isolates from farms P126, P152, P595, P646, P657 and P676. The cluster forming between four of the isolates from farm P646 (P646-1, P646-4, P646-6 and P646-7), and between four of the isolates from farm P657 (P657-2, P657-3, P65-5 and P657-8) were supported by bootstrap values at the level of 99.4 % and 100 %, respectively. The complete set of isolates from farm P657 was supported by a bootstrap value of 88.5 %.

On the cladogram of pooled data in cluster 6 it can be seen the human isolate ExRDVC clustering together with isolates from farm P595 which were also found forming a cluster with the human isolate ExRDVC in group 1 on the phylogram generated by primer AP1247 and in cluster 2 of the phylogram by AP1254. The isolates P595-3 and P595-9 pairing together in cluster 3 of the cladogram by pooled data were supported by

a bootstrap value of 100 %, and the isolates P595-1 and P595-2 also pairing together in that cluster were supported by a lower bootstrap value of 66.1 %. The two isolates from farm P152 always paired together on the phylograms of the four primers, indicating that these isolates were very closely related, this pair of isolates was supported by a bootstrap value of 100 % as seen in cluster 5 of the cladogram by pooled data. The isolate P34 was found together with isolates of either from farm P657 in cluster 4, or from farm P646 in cluster 4 on the phylograms of AP1247 and AP1254, respectively. However, that consistency was not supported by high bootstrap values as can be seen on the cladogram. In cluster 9 of the phylogram by AP002 the isolate P218-2 was found together with isolates from farm P152. In cluster 2 of the phylogram by AP1247, and in cluster 1 of the phylogram by AP1254. The isolates P218-2 and P249-5 were found clustering together with isolates from farm P152. The consistency of that cluster forming was confirmed in cluster 5 on the cladogram of pooled data. The cluster forming between the isolates from farm P676 and the isolates P4, P6, and M1 was observed in cluster 9, cluster 3, and cluster 5 on the phylograms by three of the primers AP002, AP1254 and APKG, respectively. That cluster forming was also seen in cluster 6 on the cladogram of pooled data. However, that cluster had lower bootstrap values, except for the set of isolates (P676-1, P676 –2, P676-3 and P676) that were supported by a boostrap value of 100 %. The set of four isolates from farm P126 formed two pairs, one pair formed the isolates P126-5 and P126-7, and the other pair was formed between the isolates P126-9 and P126-12 as seen in cluster 4, cluster 3 and cluster 4, and cluster 6 on the phylograms by three of the primers AP002, AP1254 and APKG, respectively. Those two pairs of isolates (P126-5 and P126-7; P126-9 and P126-12) showed consistent grouping as seen in cluster 2 on the cladogram of pooled data which were supported by bootstrap values of 100 % and 99.9 %, respectively.

As observed on the phylograms of the four primers, the two isolates from farm P249 also did not pair together on the cladogram of pooled data, thus confirming their genetic heterogeneity. Those isolates grouped together with other isolates without a clear pattern of cluster forming. The single isolates P26 and P277 were paired together in

group 2 on the cladogram and in cluster 2 on the phylogram by primers AP002 and AP1254, respectively, and were also seen together with other isolates in the cluster 5 on the phylogram of APKG. That cluster forming was consistent on the cladogram of pooled data. The pairing of isolates P26 and P277 was supported by a very high bootstrap value of 99.7 %.

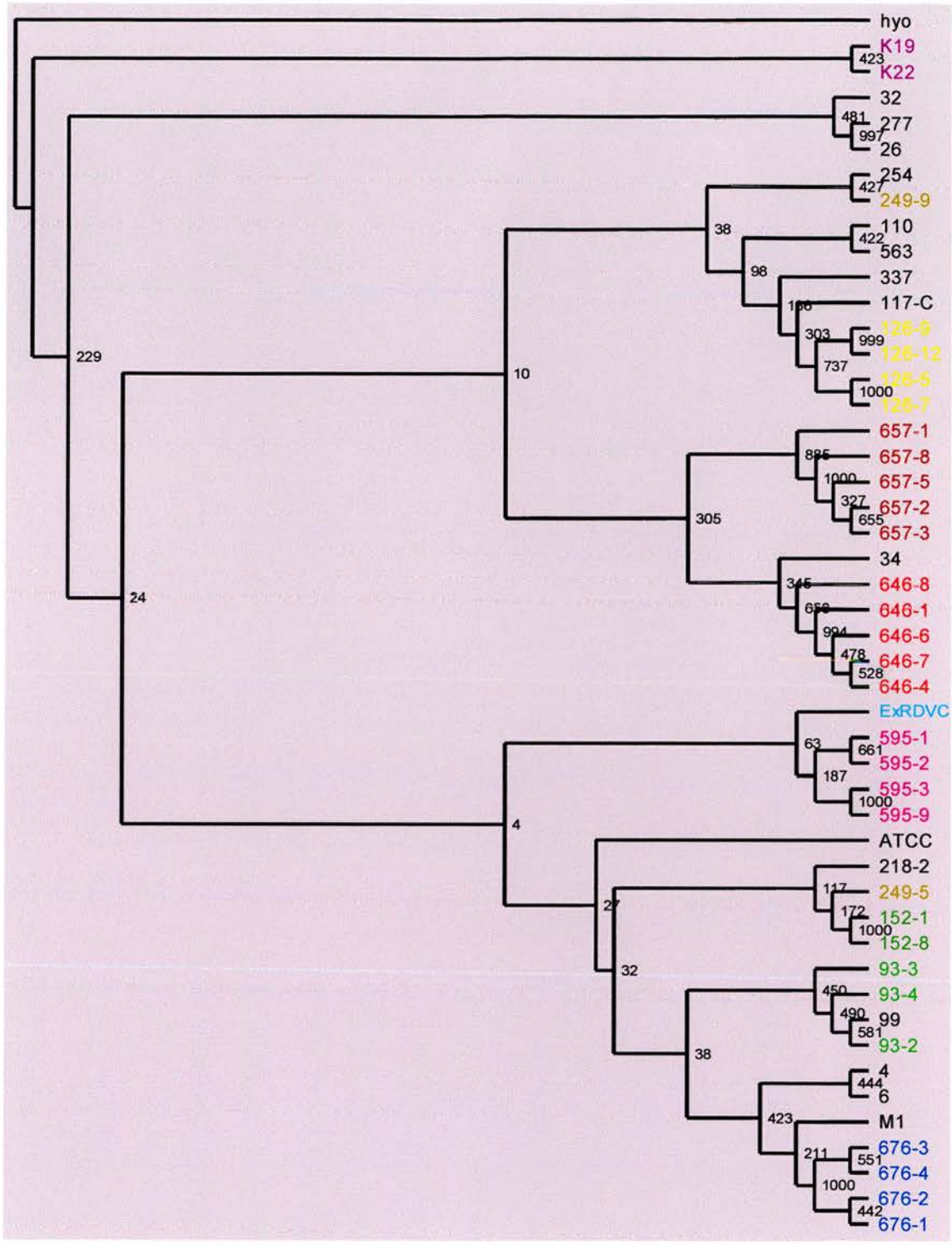

Fig. 4.9. Cladogram (1000 bootstrap) of pooled data generated by distance method showing the cluster forming of multiple isolates of *B. pilosicoli*.

Discussion

There are numerous methods of typing bacterial strains for epidemiological purposes. In this study AP-PCR was chosen because it has been used successfully for characterising isolates from disease outbreaks in other studies and has been reported as more sensitive than MEE. This method provided a measure of genetic variation between multiple isolates of *B. pilosicoli* isolates including multiple ones from the same farm. The optimisation of AP-PCR was achieved to determine the genetic relatedness of single and multiple isolates of *B. pilosicoli* without previous knowledge of their similarity. However, the isolates P4 P51/6/93, P93/2/94, P93/3/94, and P99/2/99 were previously used in a study for identification of *B. pilosicoli* by PCR (Fellström *et al,* 1997). During optimisation two of the primers were found to give inconsistent banding patterns so they were not included in the study.

Some studies have found RAPD to be more sensitive than MEE (Wang *et al*, 1993). A comparative study including 75 isolates of *E. coli* by the two methods showed that MEE for 20 diagnostic enzymes grouped the *E. coli* isolates into 15 MEE types, whereas the RAPD method using five arbitrary primers independently was able to detect intra-specific variation among those 15 MEE types. RAPDs were more informative than MEE in terms of intra-specific variation, for instance, AP D-14307 alone detected heterogeneity in 66 of the 75 isolates and the combined information from the 5 primers used detected heterogeneity in 74 of the isolates (Wang *et al*, 1993). Therefore in this study selection of primers and use of multiple primers individually helped the epidemiological differentiation of the isolates of *B. pilosicoli* which was an important consideration.

AP-PCR has been used for the genetic identification of field isolates of *B. pilosicoli* (Duhamel *et al,* 1995a). In that study a 20-mer arbitrary primer was used to characterise three field isolates. The results showed the isolates clustering together with the

reference strain P43/6/78 but were clearly separated from *B. hyodysenteriae*, validating the usefulness of this technique for the genetic characterisation of intestinal spirochaetes. The genetic differentiation between *B. hyodysenteriae* and *B. pilosicoli* could be explained by their difference in genome size since the former has a genome of 3.2 Mbp and the latter a genome of 2.4 Mbp (van der Zeijst and ter Huurne, 1997).

Because the primers used in this study produced similar information and the analysis of a total of 163 fragments (characters) contained in the pooled data set confirmed the information obtained by each primer. The results of the pooled data set in this study increased confidence and gave robustness to the genetic analysis and cluster forming of multiple isolates of *B. pilosicoli*. This is important as the probability of regaining or losing a band is unknown and is not expected to be equal so, scoring data as (0,1) format is an approximation (Welsh *et al*, 1992).

A study including field isolates of the three different species of porcine intestinal spirochaetes i.e. *B. hyodysenteriae, B. innocens* and *B. pilosicoli* using AP-PCR with two 10-mer primers used independently was carried out by Dugourd *et al* (1996). In that study, isolates of *B. innocens* and *B. pilosicoli* were more heterogeneous than *B. hyodysenteriae* isolates as the genetic distances were at the level of 0.74 to 1.13 for *B. hyodysenteriae* and 0.75 to 1.49 for *B. innocens* and *B. pilosicoli*. Isolates of serotype 9 of *B. hyodysenteriae* were more heterogeneous than isolates of serotype 8. However, it was found that multiple isolates of *B. hyodysenteriae* from the same herd could have different banding patterns and not always be clustered together as seen on the dendogram generated (Dugourd *et al,* 1996). This is in accordance with the findings in the current study where some of the multiple isolates from farms P93, P249 and P595 had higher mean genetic distances than that of isolates (unrelated) between farms, indicating high intra-specific variation. Although, the primers used in this study were similarly informative of the genetic variation of *B. pilosicoli*, the analysis of pooled data (163 fragments) was more robust in determining the relatedness of multiple isolates of

B. pilosicoli. In the study by Dugourd *et al* (1996), one of the two primers was more informative and discriminatory than the other, and also produced better results in terms of definition of the banding patterns generated. In the present study, of the four primers used, the banding patterns generated by APKG had a slightly poorer quality than those produced by the other three primers.

A range of genetic variation between multiple isolates from the same farm was observed in this study, such variation was higher in certain farms such as P249 and P93 than others. On the sequence analysis of the 16S rRNA gene of isolates of *B. pilosicoli* and other two genetic groups including *B. hyodysenteriae* and *B. innocens*, it was found that group IV was the most distinct group as it was clearly separated from the other two genetic groups (Petterson *et al*, 1996). In that study the genetic group of *B. pilosicoli* was the tightest and was supported by bootstrap a value of 100 %, however only three isolates were included in that study of which one was the reference strain ATCC-51139 (Petterson *et al*, 1996).

In another study, the genetic variation of human isolates of *B. pilosicoli* was assessed biochemically and by sequence analysis of a variable region of the 16S rRNA gene (De Smet *et al,* 1998). Only 9 out of 19 isolates were biochemically similar to *B. pilosicoli* isolated from pigs in Sweden. By sequencing analysis of a 283 bp fragment of the 16S rRNA gene the human isolates were shown to be heterogeneous as compared to the homogeneity shown on that region by seven pig isolates (De Smet *et al,* 1998).

The current study found a degree of genetic homogeneity among some of the multiple isolates, particularly multiple isolates from farms P676, P657, P152, and P126 which had bootstrap values higher than 95 %, as seen on the cladogram of pooled data. On the other hand, the genetic heterogeneity observed in other farms suggests that more than one clone might be infecting a single herd. In fact, it has been suggested that more than one type of intestinal spirochaete could be isolated from the same area or pig unit

(Hommez *et al,* 1998; Fellström and Gunnarsson, 1995). Clear determination of this needs further study of multiple isolates from single farms and investigation of herds over long periods to monitor changes in genotype.

Genetic diversity of *B. pilosicoli* has been assessed by rare cutting enzymes *Mlu* I and *Sma* I for macrorestriction analysis of multiple isolates (1-9) of pigs in Finish herds from two different geographic areas (Heinonen *et al,* 2000). The genetic diversity found in multiple isolates of *B. pilosicoli* suggested that more than one strain could be found infecting the same farm as more than one macrorestriction pattern was identified in the same herd. It was also found that the macrorestriction patterns were present in different geographic areas, possibly as result of farm to farm infection (Heinonen *et al,* 2000). The fact that different banding patterns were observed within multiple isolates of *B. pilosicoli* in the current study strongly suggests that more than one clone might be involved in this spirochaetal infection.

The heterogeneity of *B. pilosicoli* has also been determined by macrorestriction analysis using the rare cutting enzymes *Sac* II and *Sma* I followed by PFGE (Rayment *et al,* 1997). That study showed that isolates from a particular geographic region clustered together with isolates from different regions and a dog isolate clustered together with a human isolate, which suggested a possible epidemiological link. The heterogeniety of *B. pilosicoli* was also suggested from the lack of cluster forming of several bacteraemic isolates. Instead they were found clustering together with isolates from different host species. In fact, in that study species specific isolates did not show a tendency of cluster forming, and isolates from pigs, dogs, and humans were seen within the same main clusters, suggesting a possible epidemiological link between isolates of different species (Rayment *et al,* 1997). The type strain ATCC-51139 clustered together with a human isolate from a child (Rayment *et al,* 1997). Although, in the present study only two dog isolates and one human isolate were included, the phylograms (distance method and parsimony) of pooled data showed that the dog isolates paired together but were in the

same cluster as the reference strain ATCC-51139, indicating a close genetic relatedness to *B. pilosicoli* of pig origin.

Using a pig isolate of *B. pilosicoli* as a probe for hybridisation showed that 7 of 16 isolates of WBHIS from pigs and one isolate from a human hybridised with that probe, indicating a degree of genetic similarity between the isolates from different species (Ramanathan *et al,* 1993).

Atyeo *et al* (1996) showed genetic relatedness between human isolates from different countries and a dog isolate using data from PFGE. That study by Atyeo *et al* (1996) suggested that the epidemiological link between isolates of *B. pilosicoli* from different species (pig, dog, chicken and human) may be feasible since human and avian isolates were found clustering together with either pig or dog isolates. In this study, a possible epidemiological link between isolates of the trilogy pigs, dogs and humans could be suggested from the cluster 3 of group 3 on phylogram by AP1247 and from cluster 2 of group 1 on phylogram of pooled data (163 fragments), in which the type strain ATCC-51139 and the dog isolate K22 were included in the same cluster. Also the human isolate ExRDVC was sharing the same cluster with either some of the isolates from farm P657 in cluster 8 of group 3 of phylogram by AP002; farm P277 in cluster 1 of group 2 on phylogram by AP1247; farm P93 in cluster 2 of group 3 on phylogram by AP1254. In fact, isolates from one host species are able to cause disease in other host species as it has been experimentally proven (Trott *et al,* 1996a).

The primer KG clustered together the human isolate ExRDVC with the dog isolate K22 which could be interpreted as a possible epidemiological link between this pair of isolates. However, the epidemiological trilogy was not clear on the phylogram of pooled data (163 fragments) since the human isolate ExRDVC was found in a single cluster and the dog isolates paired together but clustering together with the reference strain

ATCC-51139. This demonstrates the usefulness of multiple primers for determining genetic diversity of micro-organisms by RAPD method to confirm genetic relatedness or epidemiological links between isolates. Had only the primer APKG been used in this study, the results might have been wrongly interpreted. Although, the four primers used in the current study produced similar information in terms of genetic variation of *B. pilosicoli* the latter instance was one of their discrepancies.

Koopman *et al* (1993) suggested more strongly the epidemiological link between human and dog isolates due to the genotypic similarity by restriction enzyme analysis and hybridisation with a flagellin gene, haemolysin gene and the 16S rDNA sequence. From that study, it was suggested that transmission of *B. pilosicoli* between dogs and humans was more likely than between pigs and humans on the basis of relative similarity (Koopman *et al*, 1993).

Since only one human isolate and two dog isolates were included in this study it is not possible to draw any further conclusions. Therefore analysis of larger number of isolates from pig, dog and human origin to address the questions in appropriately designed studies of genetic relatedness among *B. pilosicoli* isolates from these species is a potential area of future work.

Further support of an epidemiological link between isolates of *B. pilosicoli* from dogs, pigs and humans has been proposed as shown on a genetic study using AP-PCR data (Duhamel *et al,* 1995b). In that study, canine isolates were closely related to those of humans and pigs. In fact, one of the canine isolates, in particular isolate 16242-94 was more closely related to a pig isolate than others, although, another canine isolate 24072-93B from the same study appeared to be closely related to *B. innocens*. In this study, the human isolate ExRDVC of *B. pilosicoli* was more closely related to pig isolates than to the dog isolates, and the dog isolate K19 was more closely related to the reference strain ATCC-51139 than to the human isolate.

In the study by Duhamel *et al* (1995b) one (isolate 16242-94) out of five canine isolates showed to be closely related to the reference strain ATCC-51139 as those isolates were found pairing together in a phylogenetic tree. Interestingly, in that study isolates of human, dog and pig origin were found in the same cluster in which two of the canine isolates paired together.

The genetic diversity of intestinal spirochaetes that infect chickens seems to be high since representatives of the genetic groups *B. innocens, 'Brachyspira-Serpulina' intermedia* and *B. pilosicoli* clustered together with chicken isolates in a phylogenetic tree generated using MEE data (McClaren *et al,* 1997). Interestingly, from that study 26 out of 56 (46.0 %) of the chicken isolates were found clustering together into two new genetic groups of which one had 25 isolates, showing high heterogeneity, and the other new genetic group included only one isolate (McClaren *et al,* 1997).

In summary, AP-PCR was used successfully to differentiate *B. pilosicoli* isolates. Selection of appropriate primers and use of multiple primers enhance analysis since it account for minor differences between isolates which may arise through the use of single primers. *B. pilosicoli* was shown to be highly heterogeneous at the level examined here, however, the distribution of isolates from other origins (dog and human) among the porcine isolates suggests that genotypes are not host-specific and cross-species transmission may occur. With respect to the genotypes of pig isolates of the same farm, those isolates generally clustered together confirming their genetic relatedness, however, as none of the multiple isolates were found to be identical it suggested that infections by *B. pilosicoli* in UK farms may not be by a single (clone) genotype. This may have implications for management procedures to limit infection caused by this micro-organism.

Chapter five

EFFICACY OF SEVEN DISINFECTANT-SANITISERS AGAINST *Brachyspira pilosicoli*

Introduction

Among the intestinal spirochaete species that cause disease in pigs, *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* are the most significant. The maintenance of healthy and efficient pig unit requires good control of potential pathogens such as these since diseases can potentially occur at any stage during production cycle. Concentration of pigs in a confined area leads to multiplication and circulation of micro-organisms such as bacteria, viruses and parasites. Confinement of large numbers of pigs increases the risk of disease outbreaks in large intensive production units and the risk may be higher when premises and equipment are heavily loaded and are not clean frequently or effectively (Hinton and Bale, 1991). Methods for preventing or controlling infectious diseases in pigs are multiple and include management practices such as all-in-all-out, segregation of different age groups, medicated early weaning, off-site weaning, multiple site production, use of antimicrobial agents, and vaccination coupled with practices of cleaning and disinfection. Cleaning and disinfection of buildings are part of an integrated programme for disease control in which the target is to achieve a final bacterial count of 10^3 cfu/cm^2 (Böhm *et al*, 1998). The decreasing of potential pathogens in pig farms by cleaning and disinfection reduces the likelihood of dissemination of these type of micro-organisms, such practices promote a better environment for animal production units. Guidelines for cleaning and disinfection have been described (Waddilove and Blackwell, 1997).

The majority of pathogens that infect pigs are transmitted by in-contact pigs, i.e. pigs sharing the same pen or the immediate environment (premises, equipment) contaminated with secretions and excretions of infected animals. A survey carried out in the UK on the excretion of pathogens showed that the excretion rate of *Salmonella* spp. from an infected pig farm was less than 10^2 /ml in manure, although the total bacterial count in manure is reported to be 10^{10} bacteria/ ml (Strauch, 1991). The level of excretion of *B. hyodysenteriae* and *B. pilosicoli* by experimentally infected gnotobiotic pigs has been reported at the level of 1 x 10^5 to 2 x 10^9 bacterial cells per g of pig faeces (Neef *et al,* 1994).

The survival of potential pathogens in the environment depends on factors such as humidity, temperature, pH and the presence of organic matter. Some micro-organisms are able to survive in the environment for several months or even years.

Studies on the survival of *B. hyodysenteriae* found that this microorganism survived up to 60 days in manure when maintained at low temperature (Bale *et al*, 1993). *B. hyodysenteriae* in a lagoon effluent from an intensive pig unit keeping pigs infected with SD was still infective after 5 to 6 days for sentinel pigs which developed the disease after drinking the contaminated water (Olson, 1995).

In another study on spirochaete survival (Oxberry *et al,* 1998), various numbers of *B. pilosicoli* ranging from 10^4 to 10^7 were used as inocula into lake water and tap water. At the highest cell concentration (10^7) the porcine and human strains survived in lake water at 4 °C for 66 and 70 days, respectively. The survival of *B. pilosicoli* was shortened when kept at a temperature of 25 °C since the same numbers of bacteria (10^7) were viable just for 4 days. The viability of lower numbers of bacteria 10^4 to 10^6 in lake water was shorter than that of high numbers, the survival time at temperature of 4 °C ranged from 12 to 33 days. The survival of high numbers (10^7) of *B. pilosicoli* in tap water was between 1 and 7 days at 4 °C, whereas the survival of those numbers of bacteria was just for 1 day at higher temperature of 25 °C (Oxberry *et al,* 1998). Comparatively, *B. hyodysenteriae* had shorter survival times in both, lake and tap water, the longest survival time was 14 days in lake water when kept at 4 °C (Oxberry *et al,* 1998). These findings indicate that *B. pilosicoli* is more resistant to environmental conditions than *B. hyodysenteriae* and environmental conditions affects survival.

Similarly, in another study on the survival capacity, *B. pilosicoli* survived for a longer period than *B. hyodysenteriae* (Boye *et al,* 2001). These micro-organisms were kept in three different types of microcosms (at 10 °C in the dark): soil, soil plus 10 % pig faeces

and plain pig faeces. The survival times of *B. pilosicoli* in the three microcosms were 119, 210 and 210 days, respectively, whereas the survival times of *B. hyodysenteriae* were 10, 78 and 110 days, respectively, as demonstrated by culture on plates followed by PCR (Boye *et al,* 2001). Both agents are detrimental to the health of pigs and consequently to the productivity of pig farms. The survival of infectious micro-organisms is favoured by the presence of organic material which protects them from the environmental conditions and provides them with nutrients to perpetuate viable as potential pathogens.

As there is no information on the action of disinfectants against *B. pilosicoli*, this study aimed to test the efficacy of seven disinfectant-sanitisers of different chemical groups with and without the presence of organic matter against six field isolates and the type strain of this bacterium.

Material and Methods

Bacterial isolates.

Six field isolates (P51/6/93, P99/2/93, P99/5/93, P100/6/93, P93/2/94 and P93/4/94), and type strain ATCC 51139 of *Brachyspira pilosicoli* were used in assays for testing the sensitivity of the disinfectant-sanitisers. The field strains were isolated from cases of porcine colitis arising from outbreaks on different farms in the UK, as part of a surveillance programme conducted by the Scottish Agricultural College. Isolates had been kept in brain heart infusion (BHI) + 30.0 % glycerol frozen at -70°C and were resuscitated on blood agar (BA) [Columbia agar base No. 2, Oxoid + 5.0 % sheep blood] incubated anaerobically using gaspaks (AnaeroGen, Oxoid) at 38 °C for 5-7 days. Recovered cultures were subcultured onto fresh BA plates.

Bacterial culture and counting of bacterial cells

The propagation of *B. pilosicoli* cells was done in 10 ml volume of BHI as described in material and methods section of Chapter 4. Optical densities (at 600 nm) of the inoculated BHI broths were taken before and after incubation for monitoring the growth of bacterial cells. Washing of bacterial cells was done as described on "Material and Methods" section of Chapter 4. The washed bacteria were re-suspended in appropiate medium (see below) and used in sensitivity assays. Purity of cultures was checked by microscopic examination of Gram stained smears.

Counting of bacterial cell numbers was done as described in "Material and Methods" section of Chapter 3.

Disinfectants and diluents

Seven disinfectant-sanitisers of four different chemical groups were selected, all manufactured by Antec International Limited, UK. The main chemical components,

recommended dilution and use given by the manufacturer for each product are shown in Table 5.1.

The disinfectant-sanitisers were tested in the absence of organic matter by diluting them in sterile de-ionised water (SDW). For testing the products in the presence of organic matter, the disinfectant-sanitisers were diluted in either BHI or in a preparation of sterile pig faeces (SPF) diluted in PBS at 1:20. The dilution of pig faeces was done as described on "Material and Methods" section of Chapter 3.

Sensitivity assay

Initially, the tests were carried out in universal (25 ml) glass bottles in a 10 ml working volume. For each disinfectant-sanitiser a ten-fold dilution series (1:10 up to 1:100000) was prepared in each of the diluents described above. Then, 1 ml of bacterial suspension containing approximately 10^5 *B. pilosicoli* cells was added to 9 ml of each serial dilution of disinfectant-sanitiser. Subsequently, the tests were carried out in a working volume of 1 ml in small bijoux in which 0.1 ml of bacterial suspension was added to 0.9 ml of each serial dilution of disinfectant-sanitisers as described above. Contact times were assessed at room temperature (22 °C). The contact times between disinfectant-sanitisers and *B. pilosicoli* cells for the assays using BHI as diluent were 15, 30 and 45 minutes and for the assays using SPF as diluent 30 and 60 minutes. Then inocula (10µl) from each mix were plated out in duplicate onto BA plates, allowed to dry, and then incubated anaerobically at 38 °C for 3-4 days. Control inocula (no disinfectant) were included for each strain. Sensitivity assays were repeated at least twice on separate occasions.

Table 5.1. Description of disinfectant-sanitisers used in this study.

Chemical group	Product*	Main component	Other components	Recommended use	Recommended dilution
Quaternary ammonium *(QA group)*	Ambicide	Tertiary alkalamine (15-30%) Dialkyl dimethyl ammonium chloride (25-35%)	Detergent	Disinfectant	1:200
"	DSC 1000	Isopropanol (6%) Alkyl dimethyl benzy ammonium choride (16%)	Non-ionic surfactants	Sanitiser	1:1000 biostatic
"	HD3	Sodium metasilicate (7%) Alkyl dimethyl benzy ammonium chloride (4%)	Non-ionic surfactants	Sanitiser	1:160
Amphoteric surfactant-caustic soda *(AS-CS group)*	Heavy Duty	Amphoteric surfactant caustic soda (1-5%)	Non-ionic surfactant	Sanitiser	1:160
Tar-organic acids *(T-OA group)*	Farm Fluid	High boiling tar acids (30-45%) Acetic acid (30-40%)	Surfactant	Disinfectant	1:200
"	Long Life	High boiling tar acids (15-30%) Sulphonic acid (25-40%)	Surfactant	Disinfectant	1:250
Peroxygen *(P group)*	Virkon S	Potassium peroxomono sulphate (50%) Sodium alkylbenzene sulphonate 15%)	Anionic detergent	Disinfectant	1:200

* All product manufactured by Antec International Limited, UK

Data interpretation

The efficacy of disinfectant-sanitisers to inhibit the growth of *B. pilosicoli* was determined by eye examination of culture plates, recorded as the number of inocula where the growth was completely inhibited and expressed as percentage. The viability of strains was demonstrated by the recovery of the control (no disinfectant) inocula on BA plates.

Statistical analysis of data was performed using a non-parametric test (Mann-Whitney) for percentages on Minitab software for Windows.

Results

Disinfectant assays using SDW and BHI as diluents

No differences were found on the sensitivities to the disinfectant-sanitisers of the various field isolates of *B. pilosicoli*; in other words, all the isolates tested showed the same sensitivity to the disinfectant-sanitisers. No differences were observed between the contact times i.e. 15 minutes was enough for the disinfectant-sanitisers to kill *B. pilosicoli*. The breakpoint dilution for each disinfectant-sanitiser was determined (Table 5.2). In all the assays the viability of isolates was demonstrated by the recovery of the control (no disinfectant) inocula on BA plates.

Since all strains showed the same sensitivities to disinfectant-sanitisers (Appendix C), and contact times had no effect on sensitivities, efficacies were calculated using compound data.

Although the inocula were plated out in duplicate the total number of tests for each disinfectant-sanitiser was obtained from the number of inocula (taken as one since growth of *B. pilosicoli* on BA plates was developed or not) multiplied by the three contact times (15, 30 and 45 minutes). Therefore, the number of tests for each disinfectant-sanitiser were: Ambicide, DSC-100 and HD-3 tested with 6 isolates X 3 contact times = 18 tests each; Heavy Duty tested with four isolates X 3 contact times = 12 tests; Farm Fluid, Long Life and Virkon S tested with 5 isolates X 3 contact times = 15 tests each. So, the percentages of efficacy were proportional to the number of tests performed for each product. The length of contact time did not influence the effect of the products on *B. pilosicoli* since the efficacy observed was the same at 15, 30 or 60 minutes of contact time.

Table 5.2. Breakpoint dilution of disinfectant-sanitisers against *Brachyspira pilosicoli* in the absence or presence of organic matter after 15, 30 and 45 minutes of contact time.

Disinfectant	Chemical group	Recommended dilution	Breakpoint dilution	
			Absence of organic matter (SDW)	Presence of organic matter (BHI)
Ambicide	QA	1:200	1:10000	1:10000
DSC-1000	"	1:1000*	1:10000	1:1000
HD-3	"	1:160	1:10000	1:1000
Heavy Duty	AS-CS	1:160	1:1000	1:1000
Farm Fluid	T-OA	1:200	1:10000	1:10000
Long Life	"	1:250	1:10000	1:10000
Virkon	Peroxygen	1:200	1:100	1:100

* bacteriostatic
SDW= sterile deionised water
BHI= brain heart infusion

Effect of organic matter (BHI) on sensitivities

The efficacies of the disinfectant-sanitisers were lower when diluted in presence of organic matter (BHI) as compared to those diluted without presence of organic matter as SDW (Table 5.3). The most affected product was Virkon S showing no efficacy against *B. pilosicoli* in presence of organic matter at dilution 1:100. Interestingly, in the presence of organic matter Ambicide was the most effective showing an efficacy of 33.0 % at dilution 1:10000. At a 10-fold lower dilution (1:1000) the efficacies of Ambicide, Farm Fluid and Long Life were at the level of 100 % in the presence of organic matter, whereas the efficacies for DSC-1000 and HD-3 were slightly lower, 94.0 and 83.0 %, respectively. The efficacies of Heavy Duty and Virkon S without presence of organic matter at dilution 1:1000 had no detectable activity against *B. pilosicoli* (Table 5.3). BHI had a negative effect on the action of the disinfectant-sanitisers against *B. pilosicoli*, suggesting that organic matter may inhibit disinfection, thus assays were performed using pig faeces to simulate a field situation.

Table 5.3. Relative efficacies expressed as a percentage* (after 15, 30 and 45 minutes of contact time) of disinfectant-sanitisers tested at different dilutions against *Brachyspira pilosicoli* in the absence and presence of organic matter.

	Dilution*							
	1:10		1:100		1:1000		1:10000	
Disinfectant	SDW	BHI	SDW	BHI	SDW	BHI	SDW	BHI
Ambicide	100	100	100	100	100	100	89	33
DSC-1000	100	100	100	100	100	94	89	0
HD-3	100	100	100	100	100	83	0	0
Heavy Duty	100	100	100	100	0	0	0	0
Farm Fluid	100	100	100	100	100	100	47	0
Long Life	100	100	100	100	100	100	60	0
Virkon S	100	100	93	0	0	0	0	0

* Percentage of plates in which growth was totally inhibited
SDW= sterile deionsed water, absence of organic matter
BHI= brain heart infusion, presence of organic matter

Sensitivities to disinfectant-sanitisers using sterile pig faeces

The reference strain ATCC-51139 was included in these assays, therefore the number of strains tested were 7. The total number of tests per product was then 42 i.e. three assays per isolate-strain multiplied by 2 contact times = 42.

The breakpoint dilutions of products when diluted with SDW were 1:10000 for Ambicide, DSC-1000, HD-3, Farm Fluid, and Long Life; and 1:100 for Heavy Duty and Virkon S (Table 5.4). The breakpoint dilutions in the presence of SPF were 1:1000 for Ambicide, DSC-1000, Farm Fluid, and Long Life; and 1:100 for HD-3, Heavy Duty and Virkon S (Table 5.4). In the presence of SPF matter the breakpoint dilutions of Ambicide, DSC-1000, Farm Fluid and Long Life were reduced 10-fold, and the breakpoint dilution of HD-3 was reduced 100-fold. No change was observed in the breakpoint dilutions (1:100) of Heavy Duty and Virkon S in the presence of SPF (Table 5.2), however their efficacies were markedly affected.

Table 5.4. Breakpoint dilution of disinfectant-sanitisers against *Brachyspira pilosicoli* in the absence or presence of organic matter after 30 and 60 min of contact time.

Disinfectant	Group	Recommended dilution	Breakpoint dilution	
			Absence of organic matter (SDW)	Presence of organic matter (SPF)
Ambicide	QA	1:200	1:10000	1:1000
DSC-1000	"	1:1000*	1:10000	1:1000
HD-3	"	1:160	1:10000	1:100
Heavy Duty	AS-CS	1:160	1:100	1:100
Farm Fluid	T-OA	1:200	1:10000	1:1000
Long Life	"	1:250	1:10000	1:1000
Virkon	Peroxygen	1:200	1:100	1:100

* bacteriostatic
SDW= sterile deionised water
SPF= sterile pig faeces

The efficacies (% of plates in which growth was totally inhibited) at the highest breakpoint dilutions with SDW were: Ambicide, 43.0 % (1:10000); DSC-1000, 64.0 % (1:100000); HD-3, 0.0 % (1:10000); Heavy Duty, 86.0 % (1:100); Farm Fluid, 21.0 % (1:10000); Long Life, 50.0 % (1:10000), and Virkon S, 86.0 % (1:100). So, the highest efficacies were achieved by DSC-1000 and Long Life with levels of 64.0 and 50.0 %, respectively, and Ambicide and Farm Fluid were less efficient at the same breakpoint dilution, with efficacies at the level of 43.0 and 21.0 %, respectively. The efficacies for those four products at the previous dilution (1:1000) were 100 %.

The efficacies at the highest break point dilution in the presence of SPF were: Ambicide, 0.0 % (1:1000); DSC-1000, 14.0 % (1:1000); HD-3, 86.0 % (1:100); Heavy Duty, 7.0 % (1:100); Farm Fluid, 0.0 % (1:1000); Long Life, 14.0 % (1:1000), and Virkon S, 0.0 % (1:100) (Table 5.5). The efficacies for all the products were higher when tested without presence of organic matter (SPF) as also shown on the assays comparing BHI to SDW.

In the presence of organic matter (SPF) the best efficacies were performed again by DSC-1000 and Long Life which both still showed an efficacy of 14.0 % at the dilution 1:1000, that dilution was higher than the recommended by the manufacturer.

Table 5.5. Relative efficacies expressed as a percentage* (after 30 and 60 minutes of contact time) of disinfectant-sanitisers tested at different dilutions against *Brachyspira pilosicoli* in the absence and presence of organic matter.

	Dilution							
	1:10		1:100		1:1000		1:10000	
Disinfectant	SDW	SPF	SDW	SPF	SDW	SPF	SDW	SPF
Ambicide	100	100	100	100	100	0	43	0
DSC-1000	100	100	100	100	100	14	64	0
HD-3	100	100	100	86	100	0	0	0
Heavy Duty	100	100	86	7	21	0	7	0
Farm Fluid	100	100	100	100	100	0	21	0
Long Life	100	100	100	100	100	14	50	0
Virkon S	100	100	86	0	7	0	0	0

* Percentage of plates in which growth was totally inhibited
SDW= sterile deionised water, absence of organic matter
SPF= sterile pig faeces, presence of organic matter

Analysis of efficacy of disinfectant-sanitisers by chemical group

The breakpoint dilutions as chemical group in the absence of organic matter were: QA and T-OA groups at dilution 1:10000, and AS-CS and P groups at dilution 1:100. Whereas the breakpoint dilutions in the presence of SPF were: T-OA group at dilution 1:1000, and for QA, AS-CS and P groups at dilution 1:100 for (Table 5.6). The breakpoint dilution of T-OA group was reduced by 10-fold in the presence of SPF, whereas the breakpoint dilution of QA group was reduced 100-fold. The breakpoint dilution of AS-CS and P groups remained the same (1:100) irrespective the presence of SPF but the efficacy was reduced from 86.0 % (for both) to 7.0 % and 0.0 %, respectively.

The QA and AS-CS groups had a breakpoint dilution of 1:10000 when tested without organic matter, the efficacy at this dilution was low (36.0 %) but the efficacy at the previous dilution (1:1000) was 100 % for both groups. No change was observed in the breakpoint dilution of AS-CS and P groups when tested with or without organic matter but the presence of organic matter reduced the relative efficacy to 0.0 %.

Table 5.6. Breakpoint dilution and percentage of efficacy of four different chemical groups of disinfectant-sanitisers on seven strains of *Brachyspira pilosicoli* tested with SDW and SPF.

	SDW		SPF	
Chemical group	**Breakpoint dilution**	**Relative efficacy (%)**	**Breakpoint dilution**	**Relative efficacy (%)**
QA	1:10000	36	1:100	95
AS-CS	1:100	86	1:100	7
T-OA	1:10000	36	1:1000	7
Peroxigen	1:100	86	1:100	0

SDW= sterile deionised water, absence of organic matter
SPF= sterile pig faeces, presence of organic matter

The presence of organic matter as BHI or SPF had a negative effect on the efficacies of the disinfectant-sanitisers against *B. pilosicoli* which resulted in a statistically significant difference ($P<0.006$) between the presence of absence of organic matter when compared the percentage of plates that developed growth.

Discussion

B. pilosicoli has emerged as an important cause of disease in pigs, affecting health, welfare and productivity of farms (Taylor, 1980; Thomson *et al,* 1998, Heinonen *et al,* 2000; Moalic *et al,* 2001). The prevalence of *B. pilosicoli* infection reported in the UK as single pathogen was at the level of 25.0 %, and at the level of 27.0 % as a co-pathogen (Thomson *et al,* 1998). The organism is transmitted via faeces which contaminate the environment and consequently spread to other animals. Appropriate disinfection practices in farms are likely to limit the spread by reducing the numbers of potential infective bacteria. Since *B. pilosicoli* is an enteric pathogen therefore present in faecal material contaminating the environment the disinfectant-sanitisers were tested in presence and absence of organic matter as BHI and SPF.

This study tested the activity of a range of commercially available disinfectant-sanitisers against field strains of *B. pilosicoli.* The breakpoint dilutions were established for each product. Organic matter in the form of BHI or sterile pig faeces had an adverse effect, either reducing the breakpoint dilution of the products between 10 and 100-fold, or reducing the percentage of efficacy where no change in the breakpoint dilution was observed. The results obtained using BHI indicate that organic matter might interfere with the action of disinfectants.

Three quaternary ammonium compounds (Ambicide, DSC-1000 and HD-3), and two tar-organic acid compounds (Farm Fluid and Long Life) showed good efficacy against *B. pilosicoli* even in the presence of organic matter at higher dilutions than the recommended by the manufacturer. Heavy Duty and Virkon S are amphoteric surfactant and peroxygen compounds, respectively, which showed low efficacies at dilution 1:100 when tested with organic matter, this indicates that organic matter had more adverse effects on these products than the other two compounds tested in this study. However, their efficacies were 100 % at 1:10 (Table 5.3 and 5.5), that concentration was required to obtain 100 % efficacy against *B. pilosicoli* with those products. The concentration

required for good efficacy against *B. pilosicoli* in the presence of SPF were increased 10-fold for Ambicide, DSC-1000, Farm Fluid and Long Life; 100-fold for HD-3. The percentage of efficacy was reduced to 7.0 % for Heavy Duty and to 0.0 % for Virkon at the concentration of 1:100. Despite the adverse effect of organic matter on efficacy, the breakpoint dilutions for Ambicide and DSC-1000 (both QA group), and Farm Fluid and Long Life (both T-OA group) were still above the concentration recommended by the manufacturer. HD-3 had a breakpoint dilution 1:100 in the presence of organic matter as SPF, which was below the recommended concentration by the manufacturer, although the efficacy was still high (85.0 %) at this particular dilution.

The efficacy of Virkon S has been tested against important veterinary pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium avium* (Broadley *et al*, 1993). In that study, the maximum efficacy found was at the level of 68.0 % against *M. avium* at 30 minutes of contact time, and concentrations ranging 2.0 to 4.0 % failed to inactivate *M. tuberculosis* at 30 and 60 minutes of contact time (Broadley *et al,* 1993). However, other studies (Balavenkatasubbaiah *et al,* 1996) showed that Virkon S was 100 % effective against the spore and vegetative form of *Bacillus thuriengiensis*, and the conidia *Beaweria bassiana* at the concentrations 1:100, 1:50 and 1:25. In contrast, no efficacy against these micro-organisms was found at dilutions above 1:200 (Balabenkatasubbaiah *et al,* 1996). In agreement with this study the highest dilution of Virkon S at which activity against *B. pilosicoli* was detected was 1:100 without presence of organic matter (efficacy at the level of 86.0 %), and no activity was found at the following dilution (1:200).

Gutiérrez *et al* (1999) demonstrated the effectiveness of the quaternary ammonium compound N-Duopropenide against two of the major enterobacteria species that affect pigs (*E. coli* and *Salmonella* sp.) after one hour of contact time. However, the efficacy of this disinfectant was reduced by the presence of organic matter. Double the concentration of the product was required to eliminate these pathogens when tested in the presence of organic matter (Gutiérrez *et al,* 1999). Similar results were found in the

present study in terms of the adverse effect on the efficacy of products by the presence of organic matter.

Gutiérrez *et al* (1995) studied the efficacy of different chemical groups of disinfectants in presence of organic matter. In that study a quaternary ammonium compound showed reduction in the growth of bacteria by 94.7 % and by 99.7 % after 15 and 60 minutes of contact time, respectively. Whereas other compounds such as 3-triphenidemethane derivatives and hydrogen peroxide were either completely inactivated or showed remarkably reduced activity in the presence of organic matter. However another study by de la Puente *et al* (1998) reported ineffectiveness of quaternary ammonium compounds at low concentration against *Pseudomonas aeroginosa* in pig slurry but successful elimination of mesophilic, aerobic and anaerobic micro-organisms, and enterobacteria in filtered pig slurry when the concentration of the compound was used at 5.0 %. Other studies have also remarked on the negative effect of organic matter, reporting a two-fold increase in the concentration of products to be effective and loss of efficacy of compounds like glutaraldehyde (Isenberg *et al*, 1988).

The use of sterile pig faeces as organic matter was chosen in this study to emulate a practical situation in pig farms. It is known that poor hygiene is one of the major factors for the spread of infectious diseases, and that there is a positive correlation between high numbers of pathogen and the presence of disease (Boon and Wray, 1989). In a study on cleaning and disinfection that included 129 farms found that only 10.0 % of those farms used a detergent during damping, the majority of farms (77.0 %) applied only one disinfection, and the time between disinfection and re-stocking was 120 hrs (Madec *et al,* 1999). Application of detergents prior a disinfectant could be important to assure effectiveness of cleaning and disinfection practices since they are coupled activities. For instance, eradication of PCS was achieved by cleaning and disinfection strategies together with husbandry measures such as emptying the affected farm for few days before (during quarantine) introducing new breeding stock or the existing sows after being under a medication programme (Fossi *et al,* 2001). In that study, no isolation of

B. pilosicoli of *B. hyodysenteriae* was positive in subsequent samplings for a period of two years showing that eradication of *B. pilosicoli* was feasible using cleaning and disinfection methods coupled with husbandry measures and use of antibiotics (Fossi *et al,* 2001). However, an epidemiological study on risk factors for SD, did not find a reduction in the prevalence of the disease by the application of disinfection (Robertson *et al*, 1992). On the contrary disinfection was found to be a protective factor for the occurrence of scour problems in grower-finishing pigs (Pearce, 1999).

Among the factors that may influence the activity of disinfectants are pH, temperature, concentration, presence of organic matter or certain ions. This study on disinfectant-sanitisers showed that a contact time of 15 minutes was sufficient to inhibit the growth of *B. pilosicoli* under the laboratory conditions tested.

The evaluation on the efficacy of the disinfectant-sanitisers tested in this study was based on the total inhibition of growth of *B. pilosicoli* expressed as percentage of efficacy rather than evaluating the reduction in numbers of this bacterium. Of the seven agents tested, DSC-1000, Long Life and Ambicide performed best under the assay conditions employed.

This study demonstrates the importance of conducting susceptibility assays under conditions which most clearly reflect those *in situ* to assess which disinfectant agents may be more suitable for particular pathogens.

Chapter six

GENERAL DISCUSSION

Introduction

Porcine colonic spirochaetosis is an infectious disease causing a non-fatal diarrhoea that has an impact on the pig industry in the UK and many other countries by decreasing the growth rates of pigs (Taylor *et al,* 1980; Duhamel *et al,* 1995a). Infection in humans by *B. pilosicoli* has also been reported (Lee and Hampson, 1994; Cooper *et al,* 1986). In many countries the control of infectious diseases in pig farms include all-in all-out management systems, off site weaning, multiple site production, and the use of antimicrobials and immunogens. Some of these practices are applied to PCS, however, as the general public is becoming more concerned about the use of antimicrobials in the animal production industry, the need for alternative methods to control infectious diseases will be a challenge in the future.

The aims of this study were to determine risk factors for PCS by performing epidemiological investigations in a case-control study of data collected from visits by veterinary surgeons to pig farms, to evaluate the IMS as a method of detection of *Brachyspira* cells from faeces comparing its sensitivity with existing diagnostic methods (direct culture) and PCR, to develop an AP-PCR/RAPD technique to screen the genetic variation of single and multiple isolates of *B. pilosicoli* from single herds for epidemiological purposes and to test the efficacy of commercially available disinfectants against field isolates of *B. pilosicoli.*

Interestingly, the epidemiology of PCS is complex and involves more than one host, this is a feature that needs further attention to determine clearly the role of each species involved in the transmission of *B. pilosicoli* infection.

Epidemiological features of PCS

There are few interactions between infective agents and the environment that researchers have not considered when searching for the cause of disease. The main focus is on finding an aetiological agent rather looking for interactions between the potential pathogen and the environment, and between the different bacterial populations inhabiting the pig gut. For instance, a challenge study on gnobiotic pig showed that *B. hyodysenteriae* did not cause disease in pigs that had not had pig normal intestinal flora whereas the disease was present in pigs that had the normal intestinal flora, indicating a possible interaction between bacterial populations (Neef *et al,* 1994). It has also been postulated that the composition of the diet may play a role on *B. hyodysenteriae* and *B. pilosicoli* infections (Pluskey *et al,* 1996; Siba *et al,* 1996). Another postulated interaction of micro-organisms in the gut of pigs is between *B. hyodysenteriae* and *Fusobacterium necroforum* (Alexander *et al,* 1976). It has also been reported the presence of *Ballantidium coli* in intestinal spirochaetal infections in pigs (Hampson *et al,* 2000). There are reasons to suspect of interactions between bacterial populations and the conditions given at some point for the development of disease in which diet, management factors, herd immunity and composition of the bacterial populations structure in the pig intestine. An interesting piece of work focussed on the changes in the bacterial population structure by feeding pigs different diets, and after infection with *B. hyodysenteriae* (Leser *et al,* 1998). That study showed that the profiles of the bacterial population became destabilised due to the presence of *B. hyodysenteriae*, as a considerable number of terminal fragments of the RFLP patterns disappeared from the samples of infected pigs compared to the control pigs with no infection (Leser *et al,* 1998).

A recent study showed that *B. pilosicoli* might not be present in wild pigs as collected samples were negative on culture (Fellström *et al,* 2002). Those results may indicate that the occurrence of PCS is limited to intensive pig production and factors concerning farm management and high concentration of pigs contribute somehow to the onset of the

disease or relative susceptibility of breeds. However, other studies have demonstrated the presence of *B. pilosicoli* in remote areas infecting humans and dogs (Trott *et al,* 1997).

Viability and level of excretion of infective bacteria by affected animals may also play an important role in the epidemiology of PCS. Although the objective of this study was not focussed on levels of excretion or duration of viability of *B. pilosicoli*, it has been reported that this bacterium could survive viable for 210 days in pig faeces when kept at low temperatures (10 °C) in the dark (Boye *et al,* 2001). Therefore opportunities for horizontal transmission may be high.

The removal of infective material (faeces containing intestinal spirochaetes) from pens and buildings is essential to prevent susceptible animals becoming infected by faecal-oral contact. The removal of such material is done manually or mechanically in farms that do not have slatted floors. The frequency of cleaning varies between farms and pigs may be at risk of becoming infected by prolonged or recurrent exposure to infective material. Bearing in mind the potential of transmission of infectious diseases by direct contact with infective material slatted floors were designed to keep premises cleaner and drier to reduce the risk of faecal contamination (i.e. faeces and urine passing through the slats which reduces pig contact with such waste). Unexpectedly, in a study (Rantzer and Svendsen, 2001) to determine the risk factors for porcine proliferative enteropathy (PE) caused by *Lawsonia intracellularis*, slatted floors were identified as a significant risk factor for the occurrence of PE, whereas bedded floors were found to be protective. In the present study, slatted floors were identified to be protective for PCS ($p<0.007$), whereas solid bedded floors were significantly associated with the disease ($p<0.007$). In that study by Rantzer and Svendsen (2001) the comparison between the use of slatted floors and solid floors found that pigs housed in pens with slatted floors had lower morbidity of diarrhoea and also lower total bacterial counts than pigs allocated in premises with solid floors. Interestingly, in another study, pens with partially slatted floors were associated with enteric diseases in finishers pigs so, partially slatted floor

systems were 3.6 times greater at risk of developing enteric diseases than fully slatted floors (Pearce, 1999).

Keeping premises clean and reducing the exposure of pigs to infective material are essential factors for controlling oral-faecal transmission of infectious diseases. As it has been reported that the maximum isolation rate of porcine intestinal spirochaetes is found in faeces rather than from intestinal lesions (Jacobson *et al,* 2002), this highlights the importance of keeping pig farming operations under high standards of hygiene as this study identified solid floors as significant risk factor for the occurrence of PCS. A possible explanation is that the removal of infective material from solid floors could be less frequent so, pigs have longer exposure to that type of material.

The source of breeding stock replacements may play an important role in the epidemiology of PCS. In a study on SD, follow up investigations of 25 outbreaks showed that in 22 of those farms the source of infection was identified as carrier pigs from a farm known to be infected with *B. hyodysenteriae* (Windsor and Simmons, 1981). In this study the source of breeding stock replacement in particular farm A was identified as a risk factor for PCS. Some of the recommendations given by Windsor and Simmons (1981) to avoid the introduction of infections such as swine dysentery or PCS into pig farms by carrier animals include:

1. Veterinary surgeons should participate in the decision making before pigs are purchased
2. The veterinary surgeon should investigate whether the farm of origin has had swine dysentery or other infectious diseases which could be harmful for the destination herd, and if so, what has been done about them
3. Purchased pigs must be isolated from the main herd and observed (quarantine) for any clinical sign of disease. Sentinel pigs from the destination herd can be put in contact with the purchased pigs to act as indicators of disease. The administration of antimicrobials to the purchased pigs may be required and the route of administration will depend on the characteristics of the isolation pens. Administration of drugs can be given

in drinking water for 7 days. In the case of swine dysentery, if the disease does not appear within 30 days of arrival then the quarantine could be lifted. Cleaning and disinfection of isolation premises between batches should always be done as part of a good management routine, since SD could be transmitted by convalescent pigs after experimental infection for up to 70 to 90 days (Griffin and Hutchinson, 1980; Windsor and Simmons, 1981). So, it is possible to infect the environment by carrier animals.

IMS as improved diagnostic test

In view of the importance of carrier pigs in the epidemiology of SD and PCS (Griffin and Hutchings, 1980; Thomson *et al,* 1998), a highly sensitive diagnostic test to detect those carrier animals would help to reduce the transmission of these diseases.

It has been postulated that the incidence of *B. pilosicoli* infection may be due to the declining cases of *B. hyodysenteriae* since the dynamics of bacterial populations get affected by lower numbers of certain pathogens (Morris *et al,* 2002). It might be that other species of intestinal spirochaetes were always present in the intestinal tract of pigs but were suppressed by higher numbers of *B hyodysenteriae*, but it has also to be considered the advances in technology in terms of better diagnostic techniques for proper differentiation of bacterial species. Nowadays, better identification of bacterial species is feasible, therefore more bacterial species have recently emerged and evolutionary changes have been traced for these new emerging species.

IMS is a successful procedure for the isolation of various infectious agents from food and faecal samples such as *E. coli* and *Salmonella* spp. (Heuvelink *et al,* 1998; Widjojoamodjo, 1991; Wright *et al,* 1994). IMS for any organism requires that magnetic beads are coated with specific antibodies e.g. MAbs for a given pathogen. Many MAbs are commercially available for *E. coli* and *Salmonella*, reflecting the degree

of development. In this study, the sensitivity for recovering of *B. pilosicoli* target cells by IMS (direct method) from spiked faeces was at the level of 9.9×10^1 total viable bacterial cells when performed without washing steps (Chapter 3). These results were comparable to the sensitivity of IMS in other studies in which gastrointestinal micro-organisms were recovered from faecal samples. For example, the detection of *Shigella* spp was at the level of 10 cells per gram of faeces (Islam and Lindberg, 1992), or 100 colony forming units of *Salmonella* spp. cells per sample (Widjojoatmodjo *et al,* 1999). IMS has also been used to separate target cells from food and water samples in which much lower numbers of undesired bacteria could be interfering with the capture of target cells. Isolation of target cells from faecal samples could be more complicated due to the high numbers and wide diversity of micro-organisms present as part of the normal flora.

To enhance the viability and growth of target cells a pre-enrichment step prior to the application of IMS to samples has been reported for the isolation of *Salmonella* spp. (Stone *et al,* 1994) and *E. coli* (Chapman and Siddons, 1996). IMS using a pre-enrichment step for enhancing the isolation of *E. coli* O157 from faeces of cattle showed higher sensitivity than direct culture. Using the pre-enrichment step, the isolation rates of *E. coli* O157 for the years 1995 and 1996 were 11.1 % (30/270) and 10.0 % (27/270), respectively as compared to 1.4 % (4 /270) and 1.4 % (4/270), repsectively obtained by direct culture on selective media such as sorbitol MacConkey agar (Heuvenlink *et al,* 1998). Another study using pre-enrichment also showed superior sensitivity for detecting *E. coli* O157 by IMS as 23.2 % (33/142) of samples were positive by this method compared to 14.08 % (13/142) by direct culture, thus IMS with pre-enrichment increased the sensitivity by 9.15 % (Cubbon *et al,* 1996).

The IMS method for *B. pilosicoli* and *B. hyodysenteriae* performed in this study did not include a pre-enrichment step because the objective was to determine the sensitivity of the IMS for detection of *B. pilosicoli* and *B. hyodysenteriae* and not to assess the pre-treatment step itself. However, a pre-treatment technique for the isolation of

B. hyodysenteriae and *B. pilosicoli* from faecal samples has been attempted (Calderaro *et al,* 2001). In that study BHI was supplemented with foetal calf serum and the antibiotics spectinomycin (400 μg/ml) and rifampicin 15 μg/ml, which enhanced the growth of intestinal spirochaetes over other bacteria of the normal intestinal flora.

In the assay developed in this study some potential from the IMS results was shown when the technique was performed without the recommended washing steps. The loss of the targeted cells during the washing steps was confirmed by the recovery of *Brachyspira* cells on culture (TA plates) from inocula of the washings. By the time promising results were obtained from the IMS without washings, the reagents, in particular MAbs were insufficient to perform further assays, therefore further studies are needed to develop an IMS method that avoids loss of targeted cells. Also, more studies are needed to test the value of IMS to detect *Brachyspira* cells from field samples since the sensitivity in this study was obtained using the type strain ATCC-51139 of *B. pilsosicoli* which is likely to have become adapted to laboratory conditions. Thus IMS without washing may have potential for detecting low levels of intestinal spirochaetes in faecal samples. A possible reason for losing target cells during the washing steps might be the molarity of the washing buffer solution. However, the buffer solutions were prepared according to the manufacturer recommendation. Another possible factor that might have contributed to the instability of the MAbs might be the biological life, or the biological stability of the antibody proteins which could have been affected by transport or storage conditions.

In this study the amount of growth from inocula on direct culture was greater than that from inocula of IMS samples. This indicated that although *Brachyspira* cells were present in the inocula from IMS samples, higher numbers (larger growth) of cultured cells were obtained from control samples (without IMS) on plates. A possible explanation for that could be that *Brachyspira* cells were adversely affected by increase handling during the normal IMS procedure. Although, *Brachyspira* cells are reported to be oxygen tolerant (Baseman *et al*, 1990), aeration during pipetting for washing the

captured cells by the IMS could interfere the potential of *Brachyspira* to grow on culture.

In this study the sensitivity of the PCR for detection of *B. pilosicoli* in samples from IMS without washing was higher (9.9×10^3 total number of viable cells) than that in samples from IMS with washing (4.8×10^5 total number of viable cells).
The sensitivity of direct culture from samples of IMS without washing (9.9×10^1 total number of viable cells) was two 10-fold dilutions higher than that of samples from IMS with washing (9.9×10^3 total number of viable cells). That confirmed that more *B. pilosicoli* cells were contained in samples from the IMS without washing since both sensitivities (PCR and direct culture) were higher than those from samples of IMS with washing which was interpreted as loss of the targeted cells during washings as was confirmed by the recover of *B. pilosicoli* on culture from the washings.
As it can be seen the sensitivity of PCR for detection of *B. pilosicoli* was lower than that of culture, and the sensitivity of the PCR for detection of *B. hyodysenteriae* was similar to that of direct culture (2.5×10^1 total number of viable cells). A possible explanation of PCR being less sensitive than direct culture may be that faecal samples contain substances which inhibits PCR, for instance, polysaccharides and haem derivatives have been reported as PCR inhibitors (Monteiro *et al,* 1997). In a faecal sample the bacterial population may be very large therefore, the amount of DNA extracted before PCR is performed may contribute to cause some interference between the target DNA and the specific primers as it has been suggested by (Stacy-Phipps *et al,* 1995). So, in this study it appeared that the loss of targeted cells during the washing steps had a greater influence on sensitivity of the PCR than the potential adverse effect of faecal inhibitors contained in the IMS samples without washing, as the PCR did not seem to be inhibited by those potential inhibitors.

In this study the incubation temperature of plates inoculated with IMS and control samples was set at 42 °C rather than the usual 37 °C. One of the advantages of growing *Brachyspira* cells from faecal samples at the higher temperature is that some of the

normal intestinal is inhibited which favours the growth of intestinal spirochaetes on culture. In this study higher temperature coupled with selective culture media produced pure cultures of spirochaetes. That is an advantage as it would save time and bench work in the first instance. However by not applying IMS, it is still needed to determine whether cultures are mixed or pure, as *Brachyspira* cells usually grow as a mucoid streak on TA, it will be necessary to serial dilute the culture product in order to obtain individual colonies that can subsequently be used for species identification. If IMS techniques can be developed to avoid this laborious and technically demanding work, it would facilitate the routine isolation of *Brachyspira* spp.

Bearing in mind that PCS is transmitted by carrier animals further investigation is needed to determine the infective dose of *B. pilosicoli* to evaluate diagnostic test using numbers of bacteria close to those occurring in the field.

Genetic variation of *B. pilosicoli*

B. pilosicoli is able to infect more than one host species and genetic variation among isolates of *B. pilosicoli* from different host has been documented (Koopman *et al*, 1993; Duhamel *et al,* 1995b; Rayment *et al*, 1997). A study showed that in some instances isolates of *B. pilosicoli* from the same host species could be regarded as host specific. For instance, isolates from mice and rats had unique DNA patterns. However, this may not be the case for isolates of WBHIS from human and dogs which their genetic profile seemed to be very similar (Koopman *et al,* 1993). It may be that *B. pilosicoli* gets stabilised in a host and only then starts changing when introduced to a new host. In pig farms, rats, mice and birds share the same environment so, the probability of getting infected by each other host pathogens could be high.

A possible explanation of recurrent infections of SD and PCS is the presence of vector animals such as rats. The genetic variation of *B. pilosicoli* may also contribute to the

persistence of PCS. Due to the capacity of *B. pilosicoli* to infect more than one host, it may be possible that infection passing from one host to another (pig, dog, human, avian or rat) contribute to the genetic variation of this bacterium. The interaction of *B. pilosicoli* with different bacterial population may trigger genetic changes or gene expression which could be reflected on study of genetic variation of multiple isolates (Chapter 4).

In this study the molecular typing of *B. pilosicoli* (Chapter 4) was not compared to any other typing method. It would be interesting to run another typing method in parallel to evaluate whether the genetic diversity obtained by AP-PCR coincides with that of the other method, to give more power to the analysis. However, the binary data as (0,1) format was subjected to two methods for estimating genetic distances between isolates and there was good agreement between the two methods in terms of the cluster forming and genetic distances as displayed on the phylograms and cladograms. The results observed by the two methods gave confidence to the analysis and helped the interpretation of the genetic variation of *B. pilosicoli* isolates. Comparative studies have been done for *B. hyodysenteriae* in which MEE was compared with REA and serotyping (Lee *et al,* 1993c). In that study there was good agreement between the REA patterns, the electrophoretic types, and the serotypes, although some exceptions occurred. However, those results demonstrated the limitations of serotyping as an epidemiological tool since isolates of the same serotype may be genetically different, therefore DNA based techniques are in general more accurate and informative than the non-genetic methods used for typing of bacteria. An advantage of AP-PCR is that no previous information about the genome to be examined is needed and another advantage is its reproducibility (Williams *et al,* 1990; Welsh *et al,* 1991). Optimisation of AP-PCR can be problematic in establishing the right conditions for each primer. In this study four primers were used individually which was important in adding strength to the analysis since many of the reported studies using AP-PCR have used only one primer. Previous genetic studies using AP-PCR for the differentiation of *B. pilosicoli* isolates from pigs, dogs and humans have been done on a small number of isolates (Duhamel *et al,* 1995a).

This study assessed the genetic variation of multiple isolates of *B. pilosicoli* sampled at the same time which allowed the screening of the genetic diversity of the outbreak bacterial population. So, the genetic variation observed in multiple isolates of *B. pilosicoli* suggested that the outbreak bacterial population may not be clonal.

The crossing of species barriers by *B. pilosicoli* has been suggested from studies of isolates from humans, pigs, dogs, chickens, mice and rats genetically screened by different molecular methods (Koopman *et al,* 1993; Rayment *et al,* 1997; Atyeo *et al,* 1996). From those results the issue of inter-species transmission was raised as the isolates from the different hosts species all clustered together showing no host specific clustering pattern, and the inter-species transmission between dogs and humans was most clearly suggested (Koopman *et al,* 1993). Moreover, experimental studies have demonstrated that *B. pilosicoli* was able to cross the species barrier as isolates from humans caused significant pathological changes in the large intestine of newly weaned pigs (Trott *et al,* 1996a).

Although, in the present study only two dog isolates and one human isolate were included in the analysis, the phylogram of pooled data (163 DNA fragments) suggested that the isolates from pig and human origin were genetically related as the human isolate was found clustering together with pig isolates, showing no species specific clustering patterns. However, a study including larger number of isolates from different species would be required in order to conclude more categorically the genetic relatedness between pig, dog and human isolates.

Because the epidemiology of PCS is complex the host specificity may need further investigation. In this study multiple isolates from the same farm showed high genetic variation which in some cases was higher than the mean genetic variation of non-related isolates (between farms). The genetic typing of B. pilosicoli isolates has significance from the point of view of the potential inter-species transmission but more particularly to

establish the heterogeneity among multiple isolates from the same farm (or geographical area) to study the bacterial population structure.

Since this bacterium is able to infect various hosts species it would be interesting to investigate how long the genetics of *B. pilosicoli* within one host species remain unchanged, and if when that change occurs, it is due to genetic material exchange from bacterial populations within the same host species or from bacteria from other in-contact hosts. The genetic variation of *B. pilosicoli* may also contribute to the adaptability of this bacterium to various host species.

Control of PCS

Transmission of infective agents among susceptible animals via the environment is fairly common, however, the major route of infection for PCS is pig-to-pig contact (Pearce, 1999). By decreasing the environmental contamination the risk of disease transmission could be reduced. Measures applied for controlling PCS include the use of antimicrobials, however, in some European countries the use of antimicrobials in feed at sub-therapeutic levels has already been banned (Melin, 2001), therefore limitation for using these preventive practices may influence the control of infectious diseases. In Sweden, an approach to control postweaning diarrhoea has been attempted by given training courses to farm personnel focusing on hygiene and changes in the feeding patterns, particularly, by increasing the feed intake of lactating sows and changing from *ad libitum* to restricted feeding system for weaned pigs (Löfstedt *et al,* 2002). In that study, the courses given to farm personnel helped to decrease the incidence of postweaning diarrhoea by 50.0 % and also considerably reduced the number of farms using antimicrobials to control postweaning diarrhoea from 23.0 % (21/90) to 4.0 % (4/90) (Löfstedt *et al,* 2002). Although the control measures were not specific for PCS in the latter study, they may apply to spirochaetal infection since *B. pilosicoli* or

B. hyodysenteriae could be found in mixed infections with other enteric pathogens as found in this study (Chapter 2), and elsewhere (Thomson *et al,* 1998). There are some experimental studies that pointed out the importance of diet composition in the onset of PCS and swine dysentery (Pluskey *et al,* 1996; Siba *et al,* 1996; Hampson *et al,* 2000). The enteric diseases of pigs are complex in which more than one infectious agents are involved and also other factors such as dietary composition could contribute the onset of PCS.

Since infectious diseases in farms are costly in the immediate, mid and long term, various ways to control them have been attempted. For instance, medicated early weaning has been used for disease eradication method by breaking the cycle of infection and eliminating infectious agents such as *Mycoplasma hyopneumoniae* and *Bordetella bronchiseptica* (Alexander *et al,* 1980). However, in recent years the use of antimicrobials to control infectious diseases has had some considerations regarding multiple drug resistance in bacteria (Ladely *et al,* 2002). On the other hand, weaning pigs at early age has also had an effect on production since other "new" diseases started to emerge as a consequence of changes in the dynamics of bacterial populations within herds. As a consequence of that, in some countries the age of pigs at weaning is now legislated as part of animal welfare matters. In the UK the minimum weaning age is 3 weeks of age and in Sweden at 4 weeks of age.

In farms where off site weaning and multiple site production systems are used the weaned pigs have no contact with sows after they leave the farrowing premises. So, the point of colonisation may occur when suckling piglets are in contact with their mothers but the manifestation of infection does not occur during the suckling period. Perhaps the maternal immunity plays a role in suppressing infection, the diet may also determine the clinical presentation of PCS or it could be a matter of gut maturity and the need to develop a suitable colonic environment before disease expression occurs. As it was observed in this study the presence of other pathogens or bacterial populations may also contribute to the occurrence of PCS. Since there are no report of PCS seen in suckling

piglets further studies are needed to determine the age when colonisation occurs in relation to the clinical manifestation of infection.

The fact that *B. pilosicoli* has the capacity to survive for a long period in the environment when circumstances are adequate could make the control of this disease more difficult when cleaning and disinfection are not fully applied. The importance of thorough cleaning of pens and buildings becomes critical for controlling pathogens like *B. pilosicoli* by disinfection as was demonstrated in this study in Chapter 5. Recommendations for the all-in all-out system include leaving pens and buildings empty for minimum number of days so the pens can be thoroughly dried out to prevent any residual organism infecting incoming pigs. However, the down-time is wasted if the removal of faeces and cleaning of premises and equipment has not been done thoroughly, since the residual organic material will reduce the efficacy of disinfectants and bacteria contained in such material could infect incoming pigs.
On the other hand, the achievement of eradication of *B. pilosicoli* from an infected farm by the use of cleaning and disinfection coupled with husbandry measures (Fossi *et al,* 2001), adds weight to the importance of applying those strategies together especially for recurrent diseases such as PCS which has been reported as difficult to eradicate (Taylor and Trott, 1997).

In addition to environmental persistence of *B. hyodysenteriae* and *B. pilosicoli*, possible explanations of recurrent infections of SD and PCS are the presence of vector animals such as rats, the capacity of *B. pilosicoli* to infect more than one host species and the possibility for infection passing from one host to another. The interaction of *B. pilosicoli* within a host may trigger alterations in gene expression and/or genome structure which could be reflected in high genetic variation. However, the significance of this and any underlying mechanism remains to be defined.

Detection of carrier animals and reduction of environmental contamination by cleaning and disinfection are important issues when considering the control of PCS. The findings

of the present study clearly show the importance of hygiene and selection of appropriate disinfectant.

Future work

Improvement in sensitivity of diagnostic methods detection of porcine intestinal spirochaetes in carrier animals is still required. As mentioned above, IMS seemed promising but further work needs to be done on a system that avoids significant lost of target cells. Although, direct culture onto selective medium and incubation at 42 °C showed good sensitivity as compared to IMS (performed with the recommended washings), it still needs to be more sensitive for detecting low numbers of bacteria. As suggested by Calderaro *et al* (2001), a pre-enrichment or pre-treatment step could be included in the isolation procedure of intestinal spirochaetes, especially in laboratories lacking of molecular-based methods. Further work needs to be done to understand why there is a high loss of *Brachyspira* cells during the washing steps of the IMS. Incorporation of more than one MAb to coat magnetic beads may increase binding of target cells but the cost of this additional step has to be considered against the alternative of using molecular-based methods.

For the control of intestinal spirochaetal infections the detection of *B. pilosicoli* and *B. hyodysenteriae* by screening herds is important. It would also be interesting to study in more detail the colonisation stage (age of pigs at which colonisation occurs) in relation to the manifestation of infection. Infection may be influenced by management factors or housing design as shown in this and other studies in relation to slatted floors versus bedded (solid) floors, diet composition or interactions between bacterial populations in the intestinal tract of the pig.

The screening of breeding farms that supply breeding stock replacements plays an important role in reducing transmission. The screening could be done by PCR since the

sensitivity has been reported at the level of 250 *Brachyspira* cells per reaction (Park *et al,* 1995), which seems to be high enough to detect of *B. pilosicoli* in faecal samples.

The genetic variation of *B. pilosicoli* needs further investigation considering factors such as diet and host, age of host at which colonisation-infection occurs and the genetic profile, arrangement and gene expression in isolates of both, diseased and healthy animals from a herd affected by an outbreak of PCS. It would also be of interest to evaluate the evolution of the genetic variation in an affected farm over a long period to investigate the factors that influence the genetic changes.

PCS has been reported difficult to eradicate (Taylor and Trott, 1997). This could be due to ineffective eradication procedures or re-infection form unknown sources.
The genetic variation of *B. pilosicoli* may lead to high antigenic variation, thus making the control or eradication difficult by vaccination since antigenic variation could evolve quickly thus evading the immune response.

High genetic variation was observed in this study among multiple isolates of *B. pilosicoli* from the same farm. The reason for such genetic variation within some of the farms is unknown, and this would be an interesting area for further studies on molecular epidemiology of *Brachyspira* spp. The presence of extra chromosomal material in both *B. pilosicoli* and *B. hyodysenteriae* (Combs *et al,* 1992; Calderaro *et al,* 1998) but the role of such material in the genetics of these bacteria has not been fully investigated yet. The role of possible horizontal exchange of genetic material among this bacterial population and the mechanisms by which it occurs may also be important.

Molecular-based methods for the diagnosis of intestinal spirochaetal infections are costly. Reduction of the cost of PCR sample processing is currently done at the SAC-VIC in Edinburgh by pooling the growth of various bacteriological cultures to identify *Brachyspira* spp., so the PCR screening of a set of samples from a particular farm can be done by running just one PCR.

In conclusion, there was a significant association of the source of breeding stock replacements and solid floors with PCS; therefore, as an effort to control this disease, the pig industry must include monitoring of breeding pyramids, and careful consideration should be given to policy debates on banning slatted floors as the prevalence of the disease may increase in non-slatted floor systems. In order to control PCS and SD a highly sensitive diagnostic test is required to detect carrier pigs. IMS without the recommended washing of the targeted cells enhance sensitive of detection but further studies are required to develop method that avoids loss of targeted spirochaetes during the washing steps. The typing of multiple isolates from farms in the UK confirms the heterogeneity of *B. pilosicoli* which should be considered in epidemiological studies, for instance, tracing potential sources of infection within breeding stock suppliers or potential zoonotic infections involving pig farms, or potential transmission between various host species. Finally, hygiene of pig units by removing organic material and use of effective disinfectant product should be considered to maximise the control of *B. pilosicoli* infections.

Appendices

Appendix A

Buffers

Washing Buffer for IMS

$NaH_2PO_4 \cdot H_2O$	0.16 g
$Na_2HPO_4 \cdot 12\ H_2O$	1.98 g
NaCl	8.10 g
Distilled water to	1 liter

Adjust pH to 7.4 with sodium hydroxide

Add 0.1 %(w/v) bovine serum albumin (final concentration) to PBS

Coating Buffer for IMS

0.1 M phosphate buffer pH 7.4

$Na\ H_2PO_4 \times H_2O$ (MW 137.99)	2.62 g
$Na_2\ HPO4 \times 2\ H_2O$ (MW 177.99)	14.42 g
Dissolve in distilled water and adjust to	1 liter

Electrode running buffer (5x), pH 8.3 (600 ml)

Tris base	9 g (15 g/l)
Glycine	43.2 g (72 g/l)
SDS	3 g (5g/l)

Dilute 60 ml (5x) in 240 ml distilled water for one electrophoretic run

TAE buffer (50 X stock solution)

Tris base	242 g
0.5 M EDTA	100 ml
Distilled water	1 lt

Add 242 the Tris base to the 0.5 M EDTA solution

Adjust pH to 8.0 with acetic acid

Make up to 1 lt

5 X Loening buffer (electrode) buffer pH 7.6-7.8

Tris base	21.7 g
EDTA (di sodium salt)	1.85 g
Sodium dihydrogen orthophosphate	23.4 g
Distilled water	1000 ml

Adjust pH with concentrated HCl

TE buffer

1M tris-HCl pH 8.0	1ml
0.5M EDTA ph 8.0	1 ml

Make up to 500 ml

Gel electrophoresis

Agarose gel electrophoresis

Agarose gel 1-2 %

1. Dissolve 1-2 g agarose in 100 ml TAE buffer using a flask
2. Microwave for 1 to 2 min or until starts boiling, making sure it is fully melted
3. Cool it down at 50 °C and add ethidium bromide (0.5 μg/ml)
4. Pour into mould
5. Place comb to make the wells and leave it to set for 30 to 45 min
6. Assemble the mould with the solidified gel on the tank
7. Pour enough (1 litre) TAE buffer pH 8.0 into tank over the gel before loading the samples
8. Load 10 μl of 1 Kb DNA ladder (Gibco BRL)
9. Load 10 μl of each sample mixed with 2 μl of loading sample buffer into the wells
10. Run the gel at 70 V for 40 minutes
11. Visualisation of PCR products is done under UV light, and photographs are taken

Polyacrylamide gel electrophoresis (PAGE)

PAGels (12 %)

MiliQ water	5.5 ml
40 % acrylamide	3 ml
5 X Loening buffer	2 ml
TEMED	12 µl
10 % APS (fresh made)	70 µl

The reagents are mix in a universal tube, TEMED and APS are added at the very end to polymerase the acrylamide. Assemble the glass plates on the cassette and then the cassette on the gel pouring frame. Pour the gels in between the two glass assembled plates, put the comb at the top of gels, leave gel to set for 45 min then remove the comb and rinse the wells with distilled water. Dry the excess of water with a tissue and assemble the two cassettes on the gel electrophoresis frame. Pour 1 X Loening buffer into the tank. Load 10 µl of 1 Kb DNA ladder on each side extreme of the gels; finally, load samples. Run gels at 200 V for 30 min.

Silver staining reagents

-Fix	
Ethanol	200 ml
Acetic acid	100 ml
Distilled water	1790 ml
-Stain	
Silver nitrate	0.19 g
Distilled water	100 ml
-Developer	
Sodium hydroxide	6 g
Distilled water	2000 ml
Formaldehyde	1.5 ml (add just before use)
-Stop	
Sodium carbonate	15 g
Distilled water	2000 ml

Staining procedure

1. Remove gel and put in 100 ml fixer, shake slowly for 10 minutes then drain off the solution.
2. Stain with silver nitrate, shake slowly for 10 minutes. Rinse twice with tap water.
3. Put gel in developer solution, shake slowly for 10 minutes, then drain off the solution.
4. Put gel in stop solution, shake slowly for 10 minutes.
5. Put gel in a plastic bag.

Immunomagnetic separation technique (IMS)

Direct method procedure

1. Dilute 1 g of pig faeces in 20 ml PBS (1:20 dilution)
1. Transfer 400 µl of diluted pig faeces into a 1.5 ml Eppendorf tube
2. Add 30 µl of each dilution of the bacterial suspension test to spike the faeces
3. Add 25 µl (10^7 beads approx) of coated beads to the spiked faeces
4. Incubate at slow rotation for 1.5 hrs making sure the suspension keeps homogeneous and the beads are not sitting at the bottom of the tube
5. Collect beads using magnet
6. Wash collected beads in 500 µl of washing buffer (collecting and resuspending beads) X 4 times*. Wash beads in 200 µl of washing buffer X 1 time
7. Collect beads with magnet
8. Finally resuspend beads in 75 µl of PBS

Indirect method procedure

1. Transfer 400 µl of diluted (1:20 in PBS) pig faeces into a 1.5 ml Eppendorf tube
2. Add 30 µl of bacterial suspension test
3. Add 200 µl of of MAb to *B. pilosicoli* or to *B. hyodysenteriae* diluted 1:20
4. Incubate at slow rotation for 1 hr making sure the suspension keeps homogeneous.
5. Add 25 µl (10^7 beads approx) of magnetic beads M-450 Rat anti-Mouse
6. Incubate at slow rotation for 1 hr making sure the suspension keeps homogeneous
7. Collect and wash beads as described above on steps 6, 7,8 and 9 above
8. Plate out as described on step 9 above
9. Inoculate plates as described on step 10 above

DNA extraction

QIAGEN DNA mini kit (tissue protocol)

1. Add 180 µl buffer ATL to the tube sample
2. Add 20 µl proteinase K, vortex the tube for 10-15 sec and incubate at 56 °C for 1.5 hrs, vortex the tube occasionally during incubation to homogenised the sample
3. Add 200 µl buffer AL, vortex the tube for 15 sec and incubate at 70 °C for 10 min, centrifuge the tube briefly before opening to precipitate drops from inside the tube's lid
4. Add 200 µl ethanol (96-100%) to the sample, vortex the tube for 15 sec, centrifuge the tube briefly to precipitate drops from inside the tube's lid
5. Transfer the sample to a QIAamp spin column with a 2 ml collection tube avoiding wetting the rim, centrifuge the column at 6000 *g* for 1 min. Place the QIAamp column in a clean collection 2 ml tube and discard the filtrate contained in the used collection tube
6. Add 500 µl buffer AW1 avoiding wetting the rim, centrifuge at 6000 *g* for 1 min. Place the QIAamp column I a clean 2 ml collection tube and discard the filtrate contained in the used collection tube
7. Add 500 µl buffer AW2 avoiding wetting the rim, centrifuge at high speed (20,000 *g*) for 3 min
8. Place the QIAamp column in a clean collection tube, discard the filtrate contained in the used collection tube. Add 200 µl buffer AE or distilled water and incubate at room temperature for 1 min, then centrifuge at 6000 *g* for 1 min
9. Repeat the last step if needed
10. Transfer low volumes (i.e. 25 µl) of the extracted DNA into small tubes to store at –20°C until use

Appendix B

OD of bacterial suspensions[a] as pure cultured cells of *B. pilosicoli* or *B. hyodysenteriae.*

	OD at 600 nm WL	
	B. pilosicoli ATCC-51139	*B. hyodysenteriae* isolate P944/14/00
	2.590	2.230
	2.650	2.305
	2.500	2.320
	2.470	2.260
	2.250	2.430
	1.984	2.400
	2.075	2.095
	1.932	1.954
	2.540	2.220
	2.840	2.560
	--	2.540
	2.540	2.460
	--	2.280
	2.910	--
	2.315	2.240
N	**13**	**14**
Mean	**2.43[b]**	**2.30[b]**
S^2 (variance)	**0.305**	**0.165**
SD	**0.55**	**0.40**

[a] suspensions prepared using the harvest of 2 BA plates.
[b] the mean sample of OD values was not significantly different.

Appendix C

Band frequencies by AP-002

Band type	Molecular weight (bp)	Band frequency %
1	2034.17	91.49
2	1791.78	6.38
3	1639.06	8.51
4	1595.63	6.38
5	1496.64	19.15
6	1454.9	8.51
7	1381.45	6.38
8	1344.7	72.34
9	1293.59	23.4
10	1199.6	76.6
11	1149.32	57.45
12	999.92	19.15
13	860.79	65.96
14	828.39	42.55
15	795.5	12.77
16	780.39	25.53
17	733.76	21.28
18	707.5	44.68
19	682.01	42.55
20	639.61	4.26
21	601.2	38.3
22	565.63	6.38
23	547.19	6.38
24	524.56	46.81
25	510.83	68.09
26	499.36	38.3
27	483.42	23.4
28	473.69	17.02
29	457.84	12.77
30	436.78	27.66
31	405.66	21.28
32	378.85	4.26
33	343.1	36.17
34	326.08	38.3
35	306.68	78.72
36	297.42	12.77
37	277.07	2.13

Band frequencies by AP-1247

Band type	Molecular weight (bp)	Band frequency %
1	2441.93	4.17
2	2166.49	6.25
3	2042.46	37.5
4	1926.83	10.42
5	1808.19	22.92
6	1765.26	10.42
7	1662.36	22.92
8	1597.83	16.67
9	1544.11	31.25
10	1504.17	4.17
11	1445.71	10.42
12	1362.64	22.92
13	1334.2	12.5
14	1288.83	10.42
15	1260.26	22.92
16	1199.15	25
17	1149.14	43.75
18	1058.83	22.92
19	1013.97	33.33
20	905.58	45.83
21	850.34	58.33
22	825.75	37.5
23	795.18	66.67
24	768.95	31.25
25	746.72	29.17
26	718.35	27.08
27	689.56	41.67
28	661.25	39.58
29	642.13	25
30	619.4	35.42
31	588.94	27.08
32	570.12	25
33	550.13	27.08
34	528.57	39.58
35	504.7	43.75
36	477.29	18.75
37	458.54	39.58
38	439.86	39.58
39	416.49	43.75
40	403.22	31.25
41	381.9	8.33
42	354.65	22.92
43	342.68	4.17
44	329.32	10.42
45	290.98	8.33

Band frequencies by AP-1254

Band type	Molecular weight (bp)	Band frequency %
1	2417.21	6.25
2	2134.59	14.58
3	2022.08	25
4	1927.36	43.75
5	1808.19	60.42
6	1729.18	47.92
7	1680.97	14.58
8	1573.32	54.17
9	1474.15	37.5
10	1397.34	10.42
11	1272.88	37.5
12	1232.81	45.83
13	1172.38	6.25
14	1101.6	8.33
15	1031.69	6.25
16	929.72	16.67
17	848.44	85.42
18	804.89	4.17
19	788.39	12.5
20	766.73	10.42
21	742.14	20.83
22	714	81.25
23	693.57	54.17
24	670.8	35.42
25	651.44	22.92
26	601.77	4.17
27	579.94	12.5
28	558.4	6.25
29	534.58	10.42
30	509.03	12.5
31	494.77	16.67
32	484.81	4.17
33	462.89	8.33
34	436.59	87.5
35	427.46	6.25
36	394.15	4.17
37	372.84	18.75

Band frequencies by AP-KG

Band type	Molecular weight (bp)	Band frequency %
1	2341.02	18.75
2	2199.58	18.75
3	2025.81	10.42
4	1896.6	16.67
5	1784.73	41.67
6	1693.99	12.5
7	1627.67	50
8	1548.1	33.33
9	1459.46	4.17
10	1398.02	29.17
11	1276.87	33.33
12	1194.22	45.83
13	1159.63	47.92
14	1124.2	12.5
15	1101.74	37.5
16	1044.42	16.67
17	978.41	41.67
18	943.43	41.67
19	878.52	25
20	871.54	58.33
21	846.82	68.75
22	817.8	22.92
23	795.55	16.67
24	780.33	6.25
25	757.45	4.17
26	729.28	16.67
27	689.81	75
28	672.66	93.75
29	646.56	89.58
30	620.54	72.92
31	586.71	62.5
32	576.21	33.33
33	560.61	50
34	542.06	66.67
35	512.03	45.83
36	497.22	31.25
37	476.63	27.08
38	461.99	50
39	447.96	50
40	435.33	12.5
41	421.01	8.33
42	398.37	2.08
43	383.63	6.25
44	369.2	6.25

Sensitivity of *B. pilosicoli* isolates to disinfectant-sanitisers (diluted 10-fold) after 15, 30 and 45 minutes of contact time without presence of organic matter (SDW). + growth, - no growth.

Isolate/ strain	15 minutes																											
	Ambicide				DSC-1000				HD-3				Heavy Duty				Farm Fluid				Long Life				Virkon S			
	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5
P51/6/93	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/2/93	-	-	-	+	-	-	+	+	-	+	+	+													+	+	+	+
P99/5/93	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
P100/6/93	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+
P93/2/94	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
P93/4/94	-	-	-	+	-	-	+	+	-	+	+	+					-	-	+	+	-	+	+	+	+	+	+	+
	30 minutes																											
P51/6/93	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/2/93	-	-	-	+	-	-	+	+	-	+	+	+													+	+	+	+
P99/5/93	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+
P100/6/93	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
P93/2/94	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+
P93/4/94	-	-	+	+	-	-	+	+	-	+	+	+					-	-	+	+	-	+	+	+	+	+	+	+
	45 minutes																											
P51/6/93					-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/2/93	-	-	-	+	-	-	+	+	-	+	+	+													+	+	+	+
P99/5/93	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
P100/6/93	-	-	+	+					-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
P93/2/94	-	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+
P93/4/94	-	-	-	+	-	+	+	+	-	+	+	+					-	-	+	+	-	+	+	+				

SDW sterile de-ionised water

Sensitivity of *B. pilosicoli* isolates to disinfectant-sanitisers (diluted 10-fold) after 15, 30 and 45 minutes of contact time in presence of organic matter (BHI). + growth, - no growth.

Isolate/ strain	15 minutes																											
	Ambicide				DSC-1000				HD-3				Heavy Duty				Farm Fluid				Long Life				Virkon S			
	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5
P51/6/93	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/2/93	-	-	-	+	-	+	+	+	-	+	+	+									-	+	+	+	+	+	+	+
P99/5/93	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P100/6/93	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P93/2/94	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P93/4/94	-	+	+	+	-	+	+	+	-	+	+	+					-	+	+	+	-	+	+	+	+	+	+	+
	30 minutes																											
P51/6/93	-	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/2/93	-	+	+	+	-	+	+	+	-	+	+	+									-	+	+	+	+	+	+	+
P99/5/93	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P100/6/93	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P93/2/94	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P93/4/94	-	+	+	+	-	+	+	+	-	+	+	+					-	+	+	+	-	+	+	+	+	+	+	+
	45 minutes																											
P51/6/93					+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/2/93	-	-	-	+	-	+	+	+	-	+	+	+									-	+	+	+	+	+	+	+
P99/5/93	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P100/6/93	-	-	+	+					-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P93/2/94	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P93/4/94	-	+	+	+	-	+	+	+	-	+	+	+					-	+	+	+	-	+	+	+	+	+	+	+

BHI brain heart infusion

Sensitivity of *B. pilosicoli* isolates to disinfectant-sanitisers (diluted 10-fold) after 30 and 60 minutes of contact time without presence of organic matter (SDW). + growth, - no growth.

Isolate/ strain	30 minutes																											
	Ambicide				DSC-1000				HD-3				Heavy Duty				Farm Fluid				Long Life				Virkon S			
	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5
P51/6/93	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/2/93	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/5/93	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P100/6/93	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P93/2/94	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
P93/4/94	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
ATCC-51139	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
	60 minutes																											
P51/6/93	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+
P99/2/93	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P99/5/93	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P100/6/93	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
P93/2/94	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+
P93/4/94	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+
ATCC-51139	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+

SDW sterile de-ionised water

Sensitivity of *B. pilosicoli* isolates to disinfectant-sanitisers (diluted 10-fold) after 30 and 60 minutes of contact time in presence of organic matter (SPF). + growth - no growth.

Isolate/ strain	30 minutes																											
	Ambicide				DSC-1000				HD-3				Heavy Duty				Farm Fluid				Long Life				Virkon S			
	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^2	10^3	10^4	10^5
P51/6/93	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+
P99/2/93	-	-	+	+	-	-	+	+	-	-	+	+	-	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+
P99/5/93	-	-	+	+	-	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	-	+	+	+
P100/6/93	-	-	-	+	-	-	-	+	-	-	+	+	-	+	+	+	-	-	-	+	-	-	-	+	-	+	+	+
P93/2/94	-	-	-	+	-	-	-	+	-	-	+	+	-	+	+	+	-	-	-	+	-	-	-	+	+	+	+	+
P93/4/94	-	-	-	+	-	-	-	+	-	-	+	+	+	+	+	+	-	-	-	+	-	-	-	+	-	+	+	+
ATCC-51139	-	-	-	+	-	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	-	-	-	+	-	+	+	+
	60 minutes																											
P51/6/93	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	-	+	+	-	-	+	+	-	+	+	+
P99/2/93	-	-	+	+	-	-	+	+	-	-	+	+	-	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+
P99/5/93	-	-	+	+	-	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	-	+	+	+	-	+	+	+
P100/6/93	-	-	-	+	-	-	-	+	-	-	+	+	-	+	+	+	-	-	-	+	-	-	-	+	-	+	+	+
P93/2/94	-	-	-	+	-	-	-	+	-	-	+	+	-	+	+	+	-	-	-	+	-	-	-	+	+	+	+	+
P93/4/94	-	-	-	+	-	-	-	+	-	-	+	+	+	+	+	+	-	-	-	+	-	-	-	+	-	+	+	+
ATCC-51139	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+	+	+	+	+

SPF sterile pig faeces

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